Investigating the physiological and metabolomic effects of *Ecklonia maxima*-derived biostimulant foliar application in ameliorating the effects of heat shock in tomato plants

Unathi Dladla

DLDUNA001

Department of Molecular and Cell Biology

UNIVERSITY OF CAPE TOWN

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Supervisors

Assoc. Prof. Mohammed Suhail Rafudeen

MCB Department, University of Cape Town

Prof. Jill Magaret Farrant

MCB Department, University of Cape Town

Dr Naadirah Moola

MCB Department, University of Cape Town

Declaration

I, Unathi Dladla, hereby declare that all the work presented in this thesis is my own and that it has not been previously submitted at any other university for any other degree. I know the meaning of plagiarism and declare that all the work in this thesis, save for that which is properly acknowledged, is my own.

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Unathi Dladla

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Amen

Abbreviations:

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
B	Biostimulant control group
BCAA	Branched amino acid
BH	Biostimulant-treated heat shock group
C	Control group
Df	Dilution factor
Et	Electron transfer
Epsilon	ε
DPPH	2,2-diphenyl1-picrylhydrazyl
F_m	Maximum fluorescence
F_v	Variable fluorescence
F_v/F_m	Quantum yield of PSII
FW	Fresh weight
FRAP	Ferric reducing antioxidant power
Fe^2	Ferrous ions
Fe^{3+}	Ferric ions
GC-MS	Gas chromatography-mass spectrometry
h	Hour
H	heat shock group
HS	heat shock stress
H_2O_2	Hydrogen peroxide
OH	Hydroxyl ion radical
HPLC	High-performance liquid chromatography
KEGG	Kyoto encyclopedia of genes and genomes
MDA	Malondialdehyde
MSTFA	Methoxyamination
ROS	Reactive oxygen species
SE	Seaweed extract
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
PSII	Photosystem II
PLSDA	Partial least square analysis
w/v	Weight/volume ratio

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Abstract

Tomato (*Solanum Lycopersicum*) is a globally popular horticultural commodity with great economic importance and is highly susceptible to heat shock and heat stress. Heat shock has evidenced detrimental effects on plant viability and growth, limiting crop productivity and quality. In addition to physical damage, structural damage to plant cell walls and membranes and the overproduction of reactive oxygen species (ROS) also cause metabolic and cellular disturbances. Concurrently, there has been a growing demand for sustainable and affordable agricultural practices using eco-friendly approaches to increase the heat tolerance of crops. Biostimulants are substances or microorganisms that help improve plant growth, yield, nutrient content and quality and can also enhance plant tolerance to different abiotic stresses either as stress priming agents or mitigating the stress directly. In South Africa, commercial biostimulant manufacture is focused on the brown algae (Phaeophyta), *Ecklonia maxima* (Osbeck) which grows and is harvested along the southern Atlantic coast of Africa.

In this study, the aim was to assess and determine whether the prior foliar application of *Ecklonia maxima*-derived biostimulant on tomato plants could assist in improving the tolerance of tomato plants to subsequent heat shock stress. The focus was on the physiological, biochemical and metabolic responses of tomato plants treated with or without *E. maxima*-derived biostimulant and subject to heat shock stress. This was achieved through different plant physiological and biochemical approaches that include electrolyte leakage assay, chlorophyll fluorescence and photosynthetic pigment measurements, FRAP (ferric reducing antioxidant power) and DPPH (2,2-diphenyl1-picrylhydrazyl) measurements and lipid peroxidation and proline assays. In addition, changes in the primary metabolites of the treated tomatoes were measured using gas chromatography mass spectrometry (GC-MS) to further elucidate the metabolic pathways involved in the responses to the different treatments.

From the findings, it was shown that a prior foliar application of *E. maxima*-derived biostimulant resulted in better photosynthetic efficiency and a decrease in the amount of electrolyte leakage from plant cells when subsequently exposed to heat shock stress compared to control plants without prior biostimulant application. This indicates improved cell membrane integrity and enhanced thermotolerance of *E. maxima* treated plants in response to heat shock stress. There was also a reduction in lipid peroxidation and proline content in heat shocked plants treated prior with *E. maxima*-derived biostimulant, indicating enhanced ROS scavenging and antioxidant systems in these biostimulant treated plants.

The metabolic analysis of the shoots from heat shocked plants that were prior treated with *E. maxima*-derived biostimulant identified key sugars, organic acids and amino acids. These included phenylalanine, valine, proline, threonine, myo-inositol, citric acid, mannitol, and succinic acid. The identified primary metabolites are linked to the promotion of plant growth by increasing chlorophyll content and mitigate stress by assisting in reducing the levels of ROS in plants and improving the antioxidant defence system.

This study showed that the *E. maxima*-derived biostimulant acts as a priming agent to enhance and protect photosynthesis while improving thermotolerance to heat shock stress by directly and/or indirectly enhancing antioxidant capacity in the plants.

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Chapter 1

Problem Statement and Thesis Outline

1.1 Problem Statement

The projected global population increase to 9.7 billion by 2050 poses a significant challenge to food security, as the demand for food will escalate proportionally (Desa, 2019). This projected growth will require creative solutions to expand agricultural productivity, minimize resource use, and address environmental constraints. Strategies may include adopting sustainable agricultural practices such as improving crop yields through genetic engineering, use of artificial fertilizers, and the use of biostimulants to enhance stress tolerance in crops while protecting the environment (Bellu, 2018).

In addition to global population increase, food security is also threatened by climate change where one estimation predicts a 1.8–4.0 ℃ increase in temperature by 2100 (Hall, 2000), leading to heat stress to become the major environmental stress negatively impacting plant crop growth, yield and production (Hall, 2000). Tomato (Solanum lycopersicum) agriculture plays a crucial part to South Africa's agricultural sector, contributing to the economy and food security, however elevated temperature stress negatively affects fruit production and yield (Lohar & Peat, 1998).

There has been a growing demand for sustainable and affordable agricultural practices using eco-friendly approaches to increase heat tolerance of crops. The use of stress mitigators such as biostimulants to enhance crop resilience and higher productivity under heat stress could present a sustainable alternative to counteract the adverse effects of the ever-changing climate (Saha et al., 2021). Biostimulants are defined as products, usually containing naturally occurring substances or microorganisms, that when applied to plants, can help in mineral nutrition, abiotic stress tolerance, crops yields, and quality characteristics, potentially creating a "stress memory" for future stress (Bulgari, Franzoni & Ferrante, 2019; García-Sánchez et al., 2022; Niu et al., 2022; Nephali et al., 2020).

Biostimulants based on seaweed extracts are one of the most studied and commercialized products (Cristiano et al., 2018). This group consists of macroscopic and multicellular marine algae from several taxonomic groups, such as red, green, and brown algae. The extracts are high in bioactive compounds that can activate physiological processes in plants thereby improving their performance as they contain a range of hormones, polyphenols, polysaccharides (Franzoni et al., 2022). This beneficial action is related to their high levels of minerals, carbohydrates, proteins, amino acids, hormones, and antioxidant enzymes (Ertani et al., 2014). These extracts assist the plant through increasing leaf gas photosynthetic processes (Kulkarni et al., 2019).

The brown algae, *Ecklonia maxima (Osbeck)* (*E. maxima)* Papenfuss bamboo, grows in the southern oceans and is mainly found along the southern Atlantic coast of Africa (Anderson et al., 2003). It is locally farmed and used to produce animal feed, alginate, fertilizer, and plant biostimulants. Their extracts are commercially available in various forms and have been applied to many crops for their growth promoting effects. They consist of numerous growth regulators including auxins, cytokinin's, polyamines, abscisic acid, gibberellins and brassinosteroids that play a beneficial role in the growth stimulation on many crops (Papenfus et al., 2013). Studies have shown that certain seaweed species reduce the severity of heat stress when applied as biostimulant (Ertani et al., 2018; Goñi, Quille & O'Connell, 2018; MacKinnon et al., 2010; Omidbakhshfard et al., 2020; Rayirath et al., 2009; Shukla et al., 2019). There has been to date no study to evaluate the effects of a commercial *E. maxima* derived biostimulant in alleviating heat shock in tomatoes and to understand the molecular mechanisms through which this may occur.

1.2 Thesis Outline

This Master's study's major aim was to determine whether the foliar application of *E. maxima*-derived biostimulant on tomato plants could assist in improving tolerance to heat shock and to establish, in part, the basis for any observed thermotolerance. The specific objectives of the study were to analyse the physiological, biochemical and metabolomic changes underpinning the response to heat shock treatment with and without prior foliar application of an *E. maxima*-derived biostimulant.

The null hypothesis (H0) was that prior treatment of tomatoes with *E. maxima*-derived biostimulant before heat shock stress will not change the thermotolerance of tomatoes to subsequent heat shock stress treatment.

The alternate hypothesis (H1) was that prior treatment of tomatoes with *E. maxima*-derived biostimulant before heat shock stress may enhance the subsequent thermotolerance of tomatoes to better withstand subsequent heat shock stress.

To further interrogate these hypotheses the following **research questions** were to be addressed:

1. In order to assess tomato plant thermotolerance to heat shock stress, what are the morphological, physiological and biochemical differences between tomato plants treated with and without the foliar application of *E. maxima*-derived biostimulant and subject to heat shock stress and control treatments?

2. What metabolites are differentially abundant between tomato plants treated with and without the foliar application of *E. maxima*-derived biostimulant and subject to heat shock stress and control treatments and its relationship to thermotolerance?

The research questions posed in this Master's thesis were systematically addressed, with each chapter delving into the following:

- Chapter 2 is a general introductory chapter which gives the background for this study in the context of climate change, S. African tomato production, response of plants to heat stress before discussing biostimulants, the different types of biostimulants according to their physical and chemical properties and their role in enhancing abiotic stress tolerance in plants interactions.
- Chapter 3 examines the morphological, physiological and biochemical differences between tomato plants treated with and without E. *maxima*-derived biostimulant and under heat shock and control conditions.
- Chapter 4 investigates the metabolome of the respective tomato plants treated with and without *E. maxima*-derived biostimulant under heat shock stress and control treatments.
- Chapter 5 summarizes the main findings of the study and explores the possible role of the *E. maxima*-derived biostimulant in plant protection, the study's limitations or challenges, and future research questions.

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Chapter 2

General Introduction

2.1 Introduction

2.1.1 High temperatures and tomato production

Abiotic stress, which includes drought stress, salt stress, non-optimal temperatures, and low soil fertility are some of the main reasons for the reduction in crop production globally (Catarina C. Nievola, 2017). These factors reduce the total number of viable crops and average yields for most crops by more than 50% (Catarina C. Nievola, 2017). By the end of the 21st century, the global surface temperature will likely exceed to 2℃, as noted by the Intergovernmental Panel on Climatic Change (IPCC) (Hoegh-Guldberg et al., 2018). Furthermore, climate models suggest that there will be an increase in the frequency and duration of heatwaves where the daily maximum temperature exceeds the average maximum temperature by 5℃. This will also be accompanied by a decrease in rainfall in sub-tropical regions all of which would negatively impact crop yield and productivity (Long & Ort, 2010). These predicted increased temperatures and heatwaves will also result in the increased likelihood of heat stress events on crop plants leading to reduced crop yields, compromised nutritional quality, and increased vulnerability to pest and diseases (Fahad et al., 2017).

The South African agriculture sector contributes significantly to food security, export revenues and employs more than 860,000 in the sector, and an abundant gain from the downstream value-chains. The agriculture sector is the strength of food security and promotes export to proceeds (Dube, Khulu & Mokoena, 2025). It is predicted that in S. Africa the average monthly rise in temperature will be 2℃ by the year 2050 (USAID, 2016). Furthermore, there is particular concern for regions in Limpopo, Mpumalanga, and the Eastern Cape which are predicted to have higher annual temperatures resulting in hotter and drier conditions in addition to extreme rainfall events (Affairs, 2017). This will have significant negative implications for human health, agriculture, water resources and ecosystems.

Tomatoes *(Solanum lycopersicum L.)* are cultivated commercially in all provinces in S. Africa though the major production areas are Limpopo, Mpumalanga, and the Eastern Cape provinces (Department of Agriculture, 2019). The commercial market contributes 95% of the total tomato produced and S. African tomato consumption is set to reach 543 tons by 2026 where it is the main or common ingredient for many local dishes including the staple diet of maize meal (Department of Agriculture, 2019). There is also export of fresh tomatoes to Mozambique and Zimbabwe from S. Africa.

2.1.2 Plants response to heat stress

Heat stress (HS) is defined as an increase in temperature beyond tolerance for an unknown duration, which triggers irreversible harm in plant growth and development (Alsamir et al., 2021). Heat stress also encompasses heat shock temperature stress where there is a sudden abrupt increase in temperature. When extreme temperatures coincide with the critical stage of plant growth, heat stress becomes a limiting factor to crop productivity and adaptation (Giacomo Cocetta 1 & 2022).

Tomato plants typically grow and develop at optimum temperatures between 15℃ and 32℃; however, temperatures beyond 35℃ negatively affect their growth and development (Akhoundnejad & Dasgan, 2018; Dasgan & Akhoundnejad, 2013). Being sessile, tomatoes face erratic high temperature conditions, which adversely influence them as temperatures go beyond the optimal ranges. One particular adverse effect is that of the overproduction of reactive oxygen species (ROS) (Fernández-Crespo et al., 2022). ROS include free and non-free radicals that contain oxygen and are capable of self-regulating survival with one or more unpaired electrons. Free radicals, like the hydroxyl ion radical (OH), superoxide anion radical (O_2^-), and non-free racial species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are severely toxic to plant growth and development (Fortunato et al., 2023; Waszczak, Carmody & Kangasjärvi, 2018) (Figure 2.1). As a result, when exposed to HS, ROS are produced in different cellular parts of the plant, including the plasma membrane, mitochondria, cell wall, chloroplast, peroxisome, endoplasmic reticulum, and apoplast (Milkovic et al., 2019; Snezhkina et al., 2019).

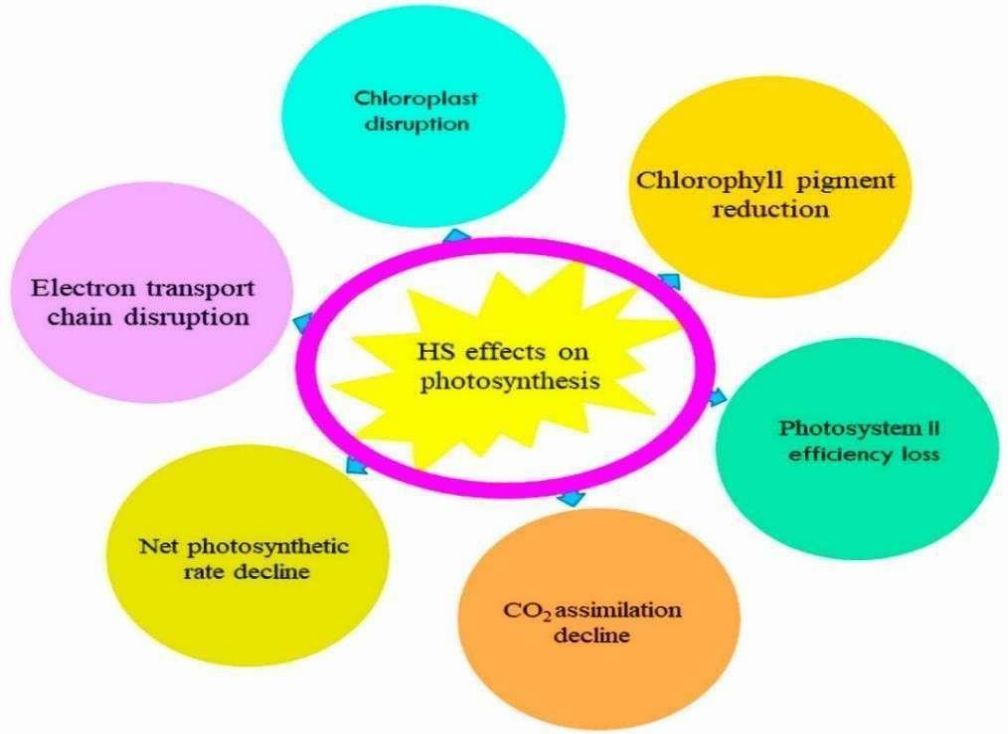

Figure 2.1: The variety of reactive oxygen species (ROS) found in tomato plants (Khan et al., 2024). ROS are a natural by-product of many plants cellular processes playing important roles in plant signaling and defence. A disruption of ROS homeostasis due to abiotic stress can lead to the overproduction of ROS which damages the plant cell.

Plant photosynthesis is very heat sensitive, and HS disrupts chlorophyll biosynthesis, CO_2 assimilation, reduces chlorophyll pigments and inactivates heat-sensitive proteins all resulting in lower photosynthetic efficiency (Zahra et al., 2023) (Figure 2.2). A study by Poudyal (2018) observed that a heat-resistant tomato cultivar showed a decline in the ratio of chlorophylls (a: b) when plants were treated with heat for 2h at 45℃ and an increase in the ratio of chlorophyll to carotenoids under control conditions of 25/20℃ (day/night). In contrast, heat-sensitive cultivars showed a decrease in the CO_2 assimilation rate (A), net photosynthetic rate (Pn), and photosystem II efficiency (F_v/F_m).

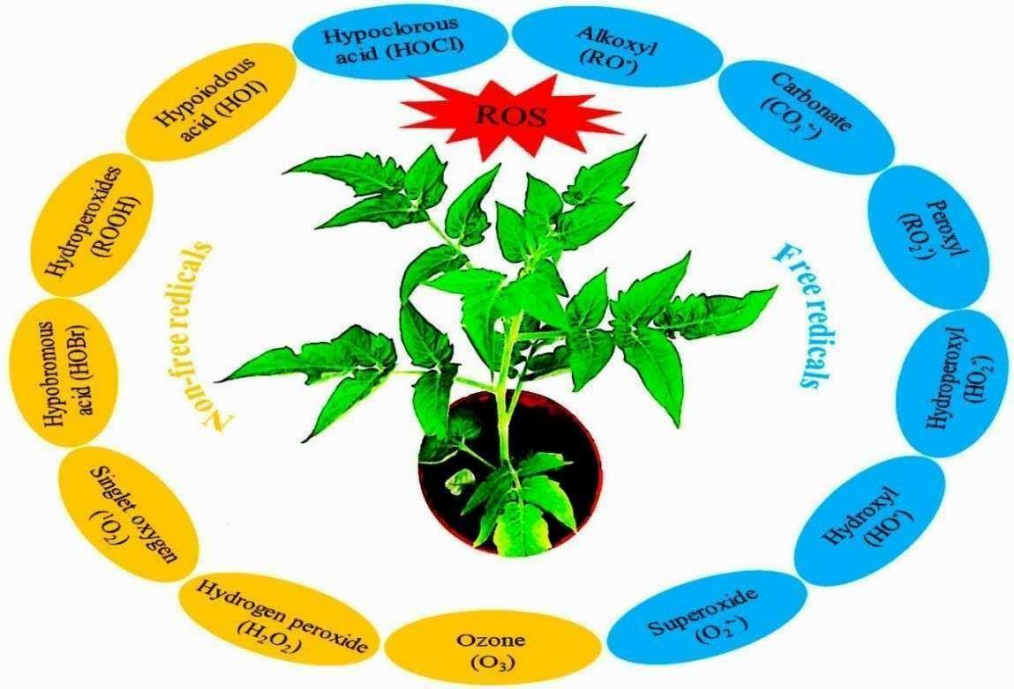

Figure 2.2: The negative impacts of heat stress on photosynthetic parameters (Khan et al., 2024). Further exposure to higher temperatures negatively impacts photosynthetic activities, thereby affecting the growth and yield of plants. Photosystem I and II (PSI, II), chlorophyll, the electron transport chain, and CO_2 assimilation are among the significant components and processes of photosynthesis (Ashraf & Harris, 2013).

2.1.3 Approaches omics technologies in plant studies for abiotic stress tolerance

Omics technologies are distinguished by their systemic investigation and analysis of extensive datasets that capture a biological systems' structure and function at specific levels; they have transformed the approaches used to study biological systems (Dai & Shen, 2022).

2.1.4 Metabolomics

Metabolomics is the investigation of low-molecular weight metabolites, including carbohydrates, fatty acids, amino acids, steroids, and lipids that play distinctive roles in interpreting the cellular biochemistry of plants (Gowda et al., 2008; Turi et al., 2018). Plant metabolites can be primary metabolites, which are crucial for growth and impact physiological processes, and secondary metabolites, which offer vital defence mechanisms in response to various stressors (Mashabela, Masamba & Kappo, 2022). A variety of advanced techniques exist for the analysis of plant metabolites (Figure 2.3), including gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography

(HPLC), paper chromatography (PC), and nuclear magnetic resonance (Mrid et al., 2021), which have proven to be valuable tools for researchers (Putri et al., 2013).

GC-MS is an analytical technique that is used to separate, identify, and quantify small volatile organic compounds and metabolites such as hydrocarbons, alcohols, aromatics, fatty acids and certain hormones in complex biological samples (Luedemann et al., 2008). GC-MS is a valuable tool for studying the metabolic responses of organisms to heat stress, which allows identification and quantification of metabolites that change under stressful conditions, providing insights into the mechanisms of heat tolerance and sensitivity (Jiang et al., 2020).

In a metabolomic study by Liu (2020), evaluating the processes and regulatory mechanisms in *Sargassum. fusiforme* in a 7-day high temperature (27℃ and 32℃), the changes in chlorophyll content and electrolyte leakage after high temperature treatment were investigated. Metabolic changes in the leaves were also analysed using GC-MS. In the results it was reported that high temperatures suppressed the chlorophyll content and increased electrolyte leakage. Furthermore, a strong modulation of various metabolisms was observed including organic acids, amino acids, sugars and sugar alcohols, esters, and amines. These changes in the metabolic pathways may contribute to the tolerance and adaptability of *S. fusiforme* to high temperature stress.

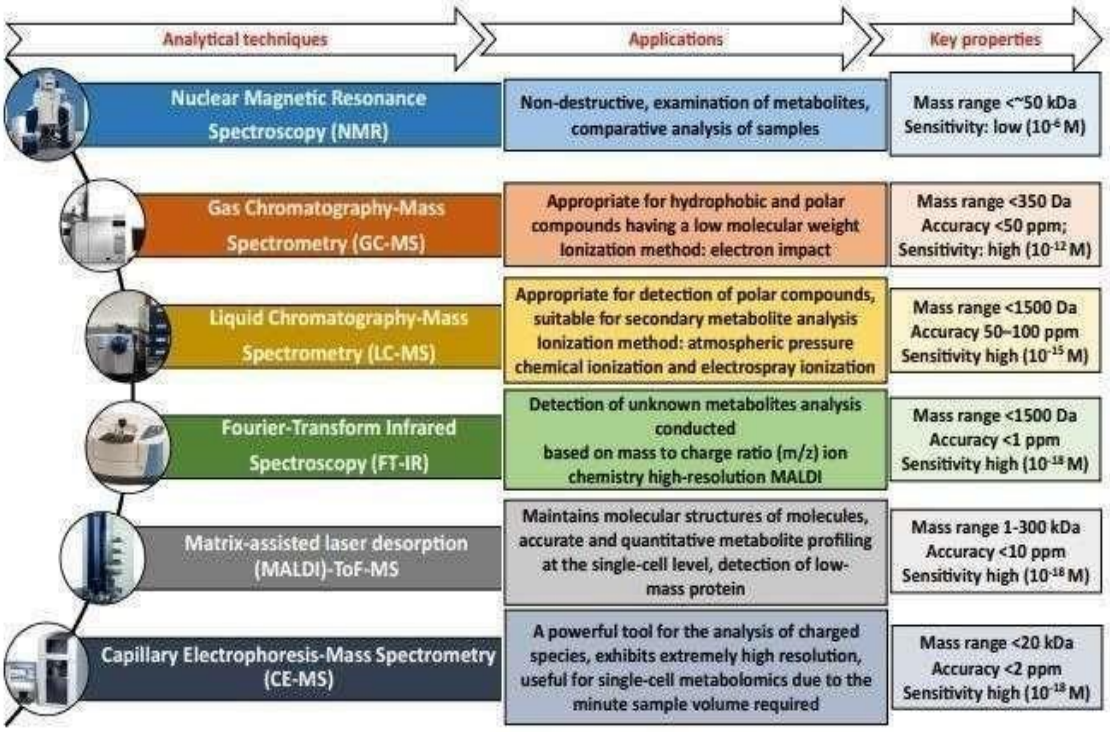

Figure 2.3: Comparison of various analytical techniques used in plant metabolomics (Raza, 2022). The various analytical techniques have different applications associated with their mass range, accuracy and sensitivity to measure specific types of metabolites.

2.1.5 Priming and plant protection

Priming, also known as stress priming or conditioning, is defined as a physiological state which involves the induction and activation of plant defences against future environmental challenges (Martinez-Medina et al., 2016). When a plant is primed, the information of the priming stimulus is stored until later exposure to the triggering stimulus (Figure 2.4) and this effect is then referred to as the stress memory in plant defence (Martinez-Medina et al., 2016). In the primed state, the resistance to counteract the stress conditions is quick and efficient (Pagano, Macovei & Balestrazzi, 2023; Pastor et al., 2013). Priming plants before exposure to stress conditions, compared to unprimed plants, reduced the harmful effects on physiology and growth, increasing their resilience (Hönig et al., 2023; Pagano, Macovei & Balestrazzi, 2023). Numerous studies have reported the effects of priming against different abiotic and biotic stresses (Conrath, 2009; Hossain et al., 2018; Martinez-Medina et al., 2016).

Based on the available literature, to enhance stress tolerance and improve seed germination, priming agents can be categorized into chemical agents, biostimulants, and nanomaterials. Each category is composed of a vast range of priming agents that are continuously increasing in number given that new studies focus on emerging priming agents (Sako, Nguyen & Seki, 2020).

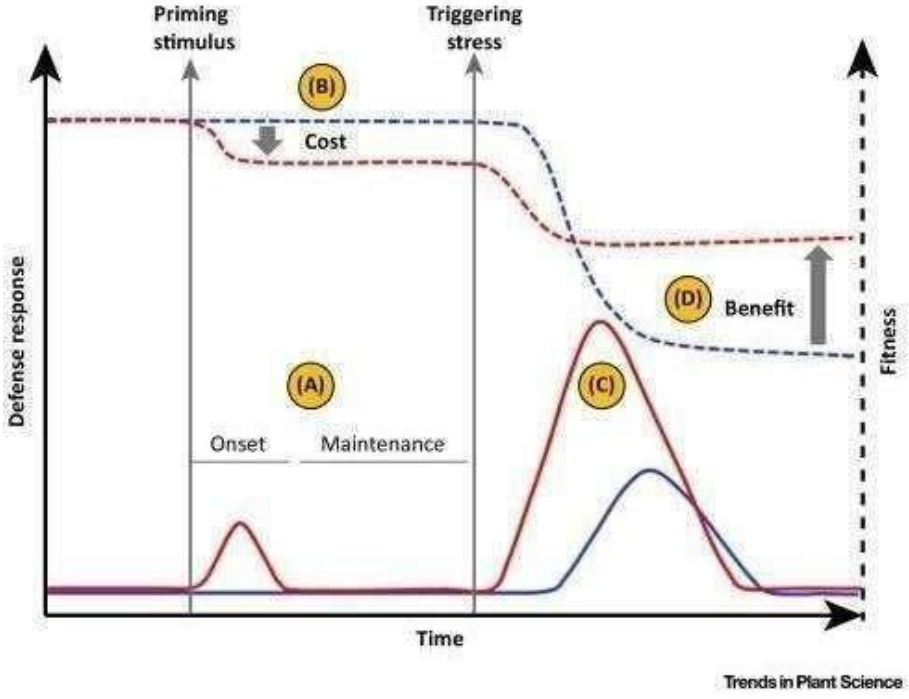

Figure 2.4: The relationship between defence responses (solid lines) and fitness (dashed lines) in primed plants (red) versus unprimed plants (blue). Analysis of defence priming requires a set of steps encompassing both the assessment of plant defences and the associated cost–benefit balance. Suggested criteria in deciding whether defence priming is present: (A) Memory: two sequential environmental events are required for establishing memory in the absence of molecular markers: the priming stimulus and the triggering stress. During priming and in the primed state (before triggering stress), plant defences are expected to be only transiently and generally faintly induced. (B) Low fitness costs: the maintenance of the primed state (before the triggering stress) has low fitness costs compared with the direct activation of defence. (C) A more robust defence response: in response to the triggering stress, primed plants mobilize cellular defences in a faster, earlier, stronger, and/or more sustained manner than do unprimed plants. (D) Better performance: primed plants are expected to defend better against a given stressor than unprimed plants. Therefore, priming enhances plant fitness in hostile environments. (Martinez-Medina et al., 2016).

2.1.6 Biostimulants and their derivatives

Plant biostimulants when applied to plants induce crucial effects that benefit plant growth and further enhance plant stress tolerance and crop characteristics (Bhupenchandra et al., 2022; Calvo, Nelson & Kloepper, 2014; Mandal et al., 2023). Plant biostimulants are any substances or microorganisms applied to plants with the aim to enhance nutrition efficiency, provide abiotic stress tolerance and improved crop quality traits, regardless of its nutrient content (Du Jardin, 2015) (Figure 2.5). These include microorganisms, plant-based extracts, seaweed extracts, humic and fulvic acids, protein hydrolysates, chitosan and other biopolymers (Sharma et al., 2014; Van Oosten et al., 2017; Zulfiqar et al., 2020). Plant biostimulants are gaining preference over chemical fertilizers and other priming agents due to their eco-friendly properties, safety, and ability to enhance plant resilience without leaving harmful residues (Van Oosten et al., 2017; Zulfiqar et al., 2020). Plant biostimulants have been shown to improve plant water use and nutrient use efficiency, improve root structure and growth while also enhancing stress and disease tolerance and facilitating induced systemic resistance (Ute,2019), (Figure 2.5).

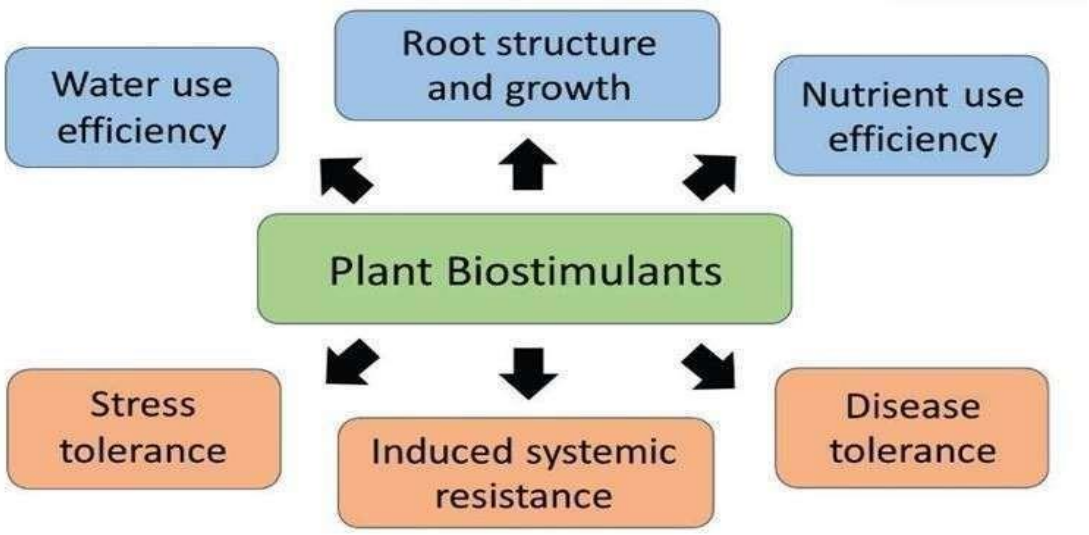

Figure 2.5: The effects of plant biostimulant on plants (Ute, 2019). These include plants with higher productivity and improved tolerance to diseases and other biotic and abiotic stresses. Other positive effects include improvement of water and nutrient uptake, improvement of water and nutrient use efficiency, improvement of root architecture and lateral root growth, and induction of systemic resistance (Calvo, Nelson & Kloepper, 2014).

2.1.7 Seaweed extracts as plant biostimulants

Seaweed extracts (SE) are derived from multicellular marine algae that form an important part of marine coastal ecosystems. They are further divided into 3 main classes based on their pigmentation, namely Phaeophyta (brown), Rhodophyta (red) and Chlorophyta (green) (Carmody et al., 2020; Kumar). The global market size and revenue generation from biostimulants derived from SE continue to increase with agricultural applications being the dominant segment of the market (Amosu et al., 2013; Blunden, 1991; El Boukhari et al., 2020; Elansary et al., 2017; Kamleshbhai et al., 2022; Pati et al., 2016; Research, Zion Market, 2025) (Figure 2.6).

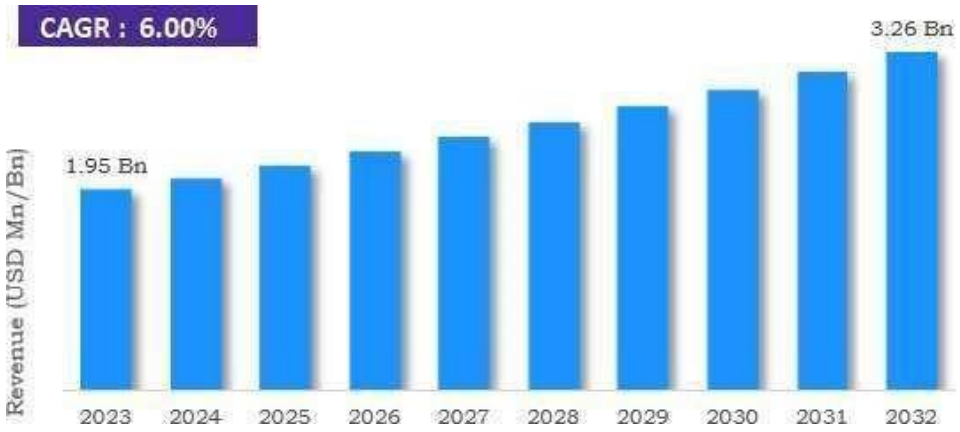

Figure: 2.6 The global SE market overview (Research, Zion Market, 2025). The global SE market size in 2023 was valued at USD 1.95 billion and is predicted to grow to around USD 3.26 billion by 2032 with a compound annual growth rate (CAGR) of roughly 6% between 2024 and 2032 (Research, Zion Market, 2025).

This growth in the SE market is also due to the growing need and requirement in agriculture for organic products to reduce or replace the excessive use of chemical fertilizers and pesticides (Research, Allied Market, 2023). SE contain an excess of bioactive compounds including, cytokinin's, auxins, abscisic acid, and gibberellins (Craigie, 2011). The anti-stress effects of SE are widely recognized in agriculture, primarily due to their rich composition of bioactive compounds that help plants cope with environmental stresses like drought, salinity, heat, and oxidative damage (Calvo, Nelson & Kloepper, 2014). Other compounds include polysaccharides, which are known to elicit plant defence responses against fungal and bacterial pathogens (Ute, 2019). It was found that presence of polysaccharides-enriched extracts in SE from six Moroccan seaweeds improved the growth, yield, and fruit quality of treated tomato plants with the metabolites SO_4, galactose, glucose, and maltose associated with the positive

effects (Mzibra et al., 2021).

Plants treated with SE have generally shown improved nutrient acquisition uptake capabilities and improved growth and development. A study performed on spinach using SE derived from *Ascophyllum nodosum* showed an increase in the biomass, protein content, chlorophyll content, flavonoids, phenolics, and antioxidant activity. The increase in biomass was correlated with an increase in the expression of the glutamine synthase (*GS1*) gene involved in nitrogen integration. The increase in the chlorophyll content was related to an increase in the expression of the enzymes betaine aldehyde dehydrogenase and choline monooxygenase (Ali, Ramsubhag & Jayaraman, 2021). It was found that treatment of tomatoes with SE derived from *Ulva lactuca* and *Padina gymnospora* improved the germination rate, seedling vigor and subsequent shoot length, root length, and weight of the treated plants (Hernández-Herrera et al., 2014). SE derived from brown algae have been shown to be rich in phenolic compounds and phytohormones, which play pivotal roles in enhancing plant growth and development (Generalić Mekinić, 2019, (Ute, 2019). Furthermore, SE play a crucial role in nutrient uptake both in hydroponic or foliar treatments (Craigie, 2011), soil conditioning, and water retention making them highly valuable in sustainable agriculture (Ute, 2019).

2.1.8 The effects of SE biostimulants in plant tolerance to abiotic and environmental stresses

SE are valuable not only for boosting plant growth but also for enhancing resilience to abiotic and biotic stresses. Many of the benefits associated with SE are due to the different bioactive compounds found in SEs and their stimulatory properties due to priming resulting in a cascade of reactions within the plant, leading to the overall growth and improvement in tolerance and/or resistance to both biotic and abiotic stress (Yakhin et al., 2017) (Figure 2.7).

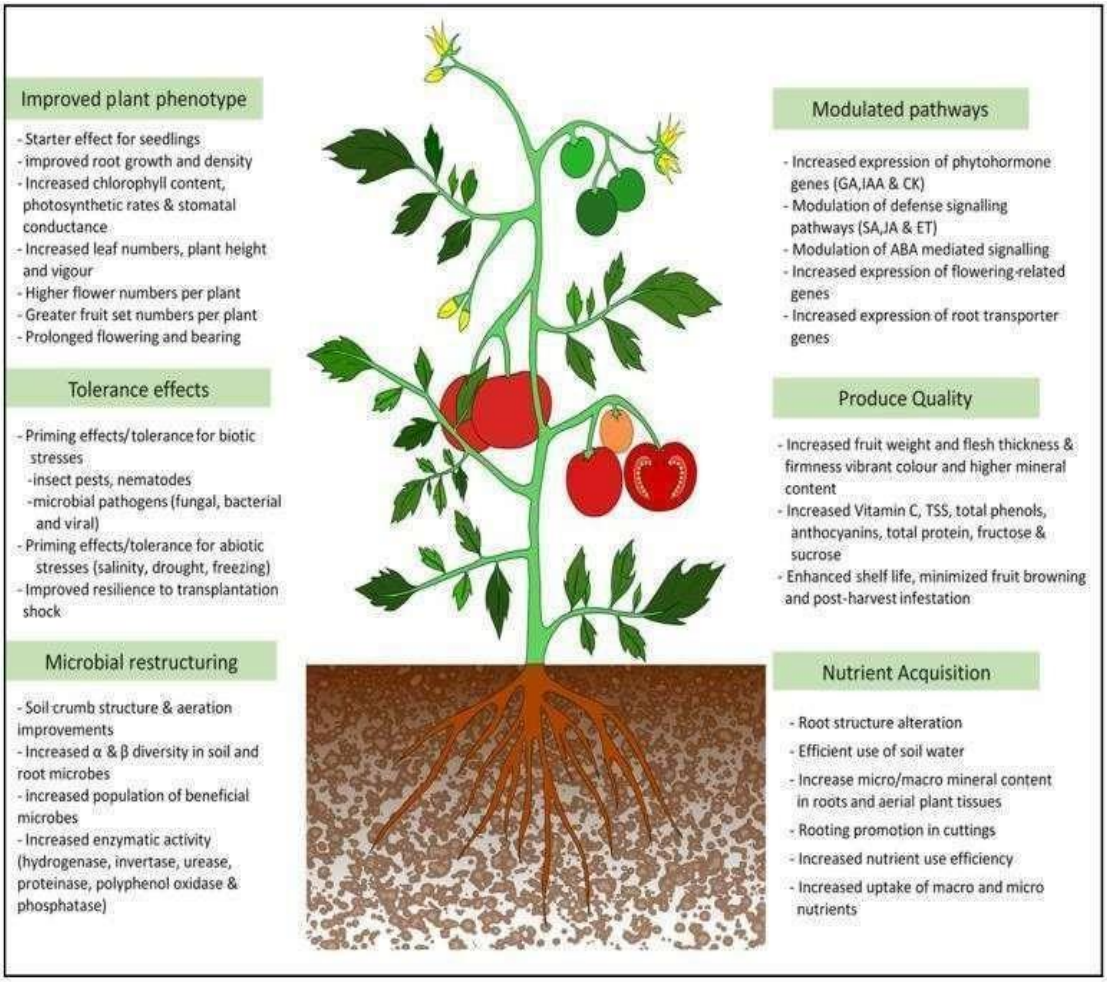

Figure 2.7: An overview of the positive effects of seaweed extracts (SE) biostimulants on plants and soil systems (Ali, Ramsubhag & Jayaraman, 2021). See text in figure for detail of the benefits associated with SE biostimulants to plant growth and stress tolerance.

SE extracts have been shown to improve tolerance to salinity, drought or both. Tomato plants showed enhanced salinity stress when treated with SE from *Padina gymnospora* compared to the control as observed by increased root and shoot length, area and weight, early flowering and enhanced fruit weight and quality (Hernández-Herrera et al., 2022). They also observed improved photosystem II (PSII) efficiency, increased chlorophyll pigment, enhanced antioxidant activity and an increase in the proline osmolyte and flavonoid content. A recent drought stress study on Italian viper bugloss (*Echium italicum* L.) treated with *A. nodosum* SE showed an increase in relative water content (RWC), electrolyte leakage (EL), total soluble sugars (TSS), free proline content, and malondialdehyde (MDA) in response to drought stress (Gheisary, Fattahi & Alipour, 2025).

The application of *A. nodosum* SE also enhanced total phenolic and flavonoid content, the

activities of ascorbate peroxidase (APX), guaiacperoxidase (GPX), and catalase (CAT) in both roots and leaves of *E. italicum,* under both normal and drought conditions. Similarly, prior treatment of tomato plants with SE derived from *A. nodosum* and then subjecting to drought stress exhibited significantly lower lipid peroxidation and higher chlorophyll content compared to untreated plants, indicating reduced oxidative damage. Additionally, these *A. nodosum* treated plants showed increased concentrations of glucose, proline, and sucrose, which are crucial osmolytes aiding in osmotic balance and stress mitigation (Goñi, Quille & O'Connell, (2018). A 2018 study by Patel showed that *Kappaphycus alvarezzi* (red algae) extract treatment of various wheat varieties under salinity and drought stress resulted in plants with increased root lengths, enhanced chlorophyll content and carotenoids, and higher tissue water content. The extract also resulted in a reduction in electrolyte leakage and lipid peroxidation, and an accumulation of osmoprotectants including proline, amino acids, and total protein (Patel, Agarwal & Agarwal, 2018).

Plants treated with SE have also been shown to mitigate against heat and cold stress. A comprehensive transcriptional, physiological and biochemical study was performed on *Arabidopsis thaliana* plants prior treated with SE derived from *A. nodosum* and subjected to heat stress (Cocetta et al., 2022). It was found that there was an increase in chlorophyll content, a reduction in the accumulation of ROS and preservation of cell membrane integrity in *A. nodosum* treated plants. Transcriptomic analysis revealed that the SE biostimulants induced heat stress-associated genes belonging to different transcription factors and heat shock protein (HSP) families. This resulted in protecting the plants from heat stress by activating specific heat shock proteins (HPS), antioxidant systems, and ROS scavengers. It was also found that *A. nodosum* SE application could mitigate long-term moderate heat stress in tomato fruit set (Carmody et al., 2020). It was observed that a particular *A. nodosum* SE formulation increased fruit number by 86% compared to untreated plants growing under heat stress conditions. There was also an increase in the accumulation of soluble sugars, and gene expression of protective HSPs in heat stressed tomato flowers before fertilization. These findings were consistent with other similar studied on SE biostimulants which showed that many of the negative impacts of heat stress can be mitigated by prior application of SE biostimulants and lead to productivity gains (Niu et al., 2022).

Treatment with *A. nodosum* SE protected *A. thaliana* plants subjected to cold stress by altering chlorophyll content (Nair et al). It was suggested that this could be possibly due to the downregulation of chlorophyll degradation genes including chloroplast stromal protein, a key regulator of cold stress tolerance. These findings suggest that SE application can effectively improve plant resilience to cold stress through coordinated genetic and metabolic adjustments (Nair et al., 2012). Though there are many studies which have shown that the application of SE biostimulant enhances the stress tolerance of crop plants to abiotic stress, it has been found that the seaweed species, its formulation, application (foliar or irrigation) and dosage contribute to its effectiveness in the field depending on the abiotic stress (Cordeiro et al., 2024).

2.5.1 *Ecklonia maxima*

The brown algae, *Ecklonia maxima (Osbeck)* (*E. maxima)* Papenfuss bamboo, is commonly found along the southern Atlantic coast of Africa (Figure 2.8) (Anderson et al., 2003). It is locally harvested and used to produce plant biostimulants where it is applied to many crops for their growth promoting effects (Sosnowski et al., 2019).

Figure 2.8: *Ecklonia maxima* (Osbeck), harvesting, processing and production. Afrikelp (Pty), Ltd is a commercial producer of the biostimulant 'Afrikelp LG1™' and other products derived from *E. maxima*. The kelp is harvested along the southern coast of S. Africa and processed, tested and formulated for both horticulture and agricultural applications (Pictures courtesy of Afrikelp (Pty) Ltd, see https://afrikelp.com/).

E. maxima derived biostimulants are known to enhance growth and yields of crops and improve the overall nutritional quality of plants. This has been ascribed to the content of the hormones from auxins and cytokinin groups. Auxins and cytokinins are plant hormones which play a crucial role in plant growth and development, with auxins promoting cell elongation and root growth, whereas cytokinins regulate cell division and differentiation, influencing shoot development and apical dominance (Digruber et al., 2018).

A previous study by Rengasamy (2015), to investigate the plant growth stimulating effects of *E. maxima* biostimulant observed improved maize growth in terms of both shoot and root elongation. Furthermore, auxin-like activity in mung bean showed an increased number of roots, shoot elongation, and seedling weight (Rengasamy et al., 2015). In another study Miceli (2021) characterised the effect of adding *E. maxima*-derived biostimulant to the nutrient solution of a hydroponic system on the growth, yield, and quality of leaf lettuce harvested during a cold storage of 21 days at 4°C. It was found that lettuce growth was enhanced, improving yield, biomass accumulation, leaf expansion, stomatal conductance, water use efficiency, and nitrogen use efficiency. *E. maxima* has also been studied for its applications in reducing the effects of both biotic (Stasio et al., 2017) and abiotic stress in terms of drought (Sabatino et al., 2023), nutrient stress (La Bella et al., 2021), and salinity stress (Rouphael et al., 2017), in crops. These studies showed the efficacy of *E. maxima* extracts as biostimulants in enhancing plant tolerance to various biotic and abiotic stresses, thereby contributing to sustainable agricultural practices. However, research on the potential benefits of *E. maxima*-derived biostimulants to mitigate abiotic stress, particularly heat stress, are few compared to other seaweed species such as *A. nodosum*.

There is increasing commercial interest in SE biostimulants given their wide-range benefits in promoting plant growth, stress resilience, and soil health (Research, Allied Market, 2023). This has facilitated further research into understanding the functional and mechanistic basis for the positive benefits associated with SE biostimulants.

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Chapter 3

Analysis of the physiological, morphological and biochemical responses in tomato *(Solanum lycopersicum)* to *E. maxima*-derived biostimulant application and heat shock treatment

3.1 Introduction

Heat stress causes varied, and often adverse changes in plant growth, development, physiological processes, and yield. Exposure to high temperatures often results in decreased chlorophyll content, decreased photosystem II (PSII) photochemistry, and decreased antioxidant enzyme activity as chloroplasts, mitochondria and plasma membranes in particular are highly sensitive to heat stress (Zeng et al., 2021). Additionally, a major consequence of heat stress is oxidative stress, caused by an increase in ROS, which damages cellular components like proteins, lipids, and DNA. Many studies have reported the negative impacts of heat stress on key enzymes involved in photosynthesis and respiration (Scafaro et al., 2021), where excess ROS can severely damage heat-stressed plant cells by disrupting multicellular components and metabolic functions (Zeng et al., 2021). Previous studies have shown that tomatoes exposed to extreme temperatures, display increased oxidative stress resulting in reduced plant growth, decreased fruit yield, impaired photosynthesis (Tonhati et al., 2020). Plants induce and/or enhance various stress protection mechanisms to assist in tolerating high temperatures. By enhancing their stress tolerance mechanisms, plants are better equipped to cope with heat stress and maintain growth, to a point, even under extreme conditions. As mentioned in Chapter 2 (Section 2.5), biostimulants are agronomic products that have been an important component in agriculture and can be used to improve the efficiency of mineral nutrition, resistance to abiotic stress and improve crop size and yield (Du Jardin, 2015).

The aim of this chapter was to investigate specific physiological, morphological and biochemical changes of tomato plants subjected to heat shock (HS) treatments for 4 h after priming with the seaweed based biostimulant, *E. maxima*. This was then followed by measurements of electrolyte leakage, chlorophyll content and antioxidant enzyme activity for the various treatment groups.

3.2 Materials and Methods

3.2.1. Plant material and growth conditions

Tomato Money Maker seeds (Starke Ayres) were sown in plastic containers in a universal peat moss blend soil. Five seeds per container were sown to allow for a selection of identically sized seedlings at the fourth leaf appearance. The experiment was carried out in a growth room under controlled conditions (16 h light/8 h dark light cycle) with a temperature fluctuation in the growth room of (26.6-28.2℃) and 41.5-43.6% relative humidity.

Once the seeds had sprouted, they were watered on alternative days, three times a week, with 100 mL tap water. At the fourth true leaf appearance, the plants were planted in individual pots (9 cm diameter, 500 mL volume) and allowed to grow until they reached a height of 30-40 cm. Subsequently, they were divided into the following four treatment groups: (1) water control (C), (2) heat shock (H), (3) *E. maxima*-derived biostimulant control (B) and (4) biostimulant + heat shock (BH). Each treatment was comprised of 6 plants each, with a total of 24 plants for the experiment (Figure 3.1 B).

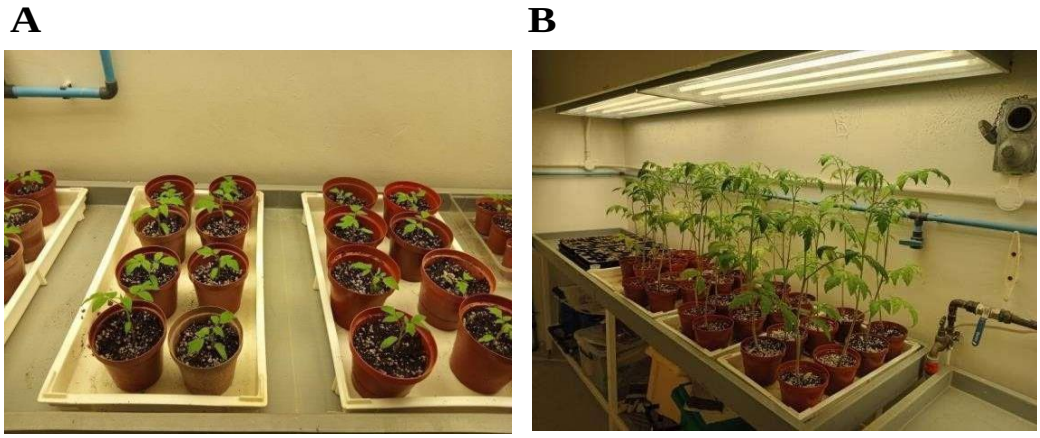

Figure 3.1: (A) Tomato plants grown at the fourth leaf stage of growth. (B) Tomato plants divided into the following four treatments, (1) water control (C), (2) heat shock (H), (3) *E. maxima*-derived biostimulant control (B) and (4) *E. maxima*-derived biostimulant and heat shock (BH).

3.2.2. Heat shock (HS) treatments

Once the plants had reached the appropriate heights of 30-40 cms, the four treatments were placed under controlled temperature conditions of 24℃ to acclimatize. This was done for the next 3 consecutive days. Thereafter, a 1% v/v solution containing the *E. maxima* biostimulant (Afrikelp^{TM}, Afrikelp (Pty) Ltd, Cape Town, S. Africa) was foliar sprayed for 2 consecutive days, on the B and BH treatments. The C and H treatments were foliar sprayed with tap water.

On the 3rd day, H and BH treatments were placed in a RUMED growth chamber, CPS-P210 climate cabinet, which is used for the simulation of standard climates and extreme environmental conditions, under the following conditions of 40°C, 100% light, 45% humidity. The C and B treatments were left under control conditions in the controlled growth room. After one hour (h) of acclimatization, the H and BH treatment were subjected to HS for 4 h under 40°C in the RUMED chamber (Figure 3.2).

Figure 3.2: Tomato plants in a RUMED growth chamber after *E. maxima*-derived biostimulant treatments in preparation for the heat shock [HS] treatments. Tomato plants were divided into the following four groups, (1) water control (C), (2) heat shock (H), (3) *E. maxima*-derived biostimulant control (B) and (4) *E. maxima*-derived biostimulant and heat shock (BH). The H and BH treatments were placed in a RUMED chamber for heat shock stress treatments.

3.2.3 Sampling procedures

Immediately after the HS treatments, the four treatments of plants were collectively measured for their physiological parameters. The chlorophyll content of the leaves was measured by assessing the chlorophyll fluorescence and the quantum yield (F_v/F_m) of light- and dark-adapted plants. The plants were then used to measure the electrolyte leakage concentrations. The shoot and root lengths were measured manually using a ruler.

The sampling involved collecting leaves from the same position on each plant. The leaves were collected in 15ml falcon tubes, before being flash frozen in liquid nitrogen. For metabolic

analysis, at least 12 g of shoot material was collected. A second leaf from the same biological replicates of the four groups was collected in 15 mL falcon tubes to be used for metabolic analysis and then flash frozen. The leaf material was then ground into fine powder by mortar and pestle using liquid nitrogen and thereafter placed in the -80 freezer to be further used for biochemical and metabolomic analysis.

3.2.4 Analysis of physiological parameters:

3.2.4.1 Photosynthetic pigment estimation

The measurement of the photosynthetic pigments was carried out according to López-Hidalgo et al. (2021). Approximately 0.1 g of fresh leaf sample was taken from each treatment and homogenized in 2 mL methanol (80%). The samples were placed in a shaker at 13, 000 rpm for 1 h at 4℃. The samples were then centrifuged at 12,000 × rpm for 5 min. About 200 µL of the supernatant was measured at 663, 645 and 480 nm. Photosynthetic pigment contents were calculated from the equations as described by Lichtenthaler, (1987) and expressed in µmol µg/mg FW of plant extract:

- chlorophyll A: 12.25_{663nm}- 2.79_{645nm}
- chlorophyll B: 21.50_{645nm}- 5.10_{663nm}

3.2.4.2 Chlorophyll fluorescence

Chlorophyll fluorescence was measured using a FluorPen FP 110 (Photon Systems Instruments, Czech Republic), a portable battery-powered fluorometer designed to assess photosynthetic efficiency in plants. Leaves were dark-adapted for 15–30 minutes using detachable leaf clips, allowing all photosystem II (PSII) reaction centres to open fully. After dark adaptation, the FluorPen probe was placed on the leaf surface, and a saturation pulse was applied to measure the maximum photochemical efficiency of PSII, the F_v/F_m ratio. The FluorPen FP 110 automatically calculated the F_v/F_m ratio, where F_v (variable fluorescence) is the difference between maximum fluorescence (F_m) and minimum fluorescence (F_o). The measurements were conducted according to the FluorPen FP 110 user manual.

3.2.4.3 Electrolyte leakage

Electrolyte leakage was conducted according to the protocol of Sherwin & Farrant, (1996) to evaluate membrane integrity under stress conditions. Fully expanded leaves were harvested from two biological replicates per treatment, with six leaves of similar size selected from each plant leaf disc (1 cm in diameter) excised from these leaves using a sterile cork borer.

The measurement of the electrolyte was done using a CM 100-2 Multiple Cell Conductivity Meter, a device used to measure electrolyte leakage in plant tissues, further assesses cell membrane damage by detecting changes in conductivity of a solution bathing the tissues after stress, such as HS.

A 100-well plate was filled with 2 mL of deionized water per well, and blank conductivity readings recorded to establish background conductivity levels. Leaf discs were then placed into the wells and allowed to soak for 1 h at room temperature, after which conductivity was measured. Following conductivity measurements, the leaf discs were removed from the wells and dried in an oven at 70 ℃ for 48 h to obtain dry weight (DW). The electrolyte leakage was calculated using the following formula:

Electrolyte leakage (µS/g DW) = (sample rate - blank rate) x 3 mL)/g DW

Since maximum possible leakage was not determined by freezing the samples in liquid nitrogen, the results represented absolute ion leakage per gram of dry weight rather than a percentage of total membrane rupture.

3.2.5 Biochemical assays

3.2.5.1 Lipid peroxidation: malondialdehyde (MDA) assay

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) content, following the method described by Campobenedetto et al. (2021) with minor modifications. Leaf material (0.1 g) was ground in liquid nitrogen and added to 1 ml 0.1% (w/v) TCA solution. The extract was centrifuged at 13,500 × rpm for 10 min at 4℃. A supernatant of 200 µL was obtained and added to 500 µL of 0.5% (w/v) TBA in 20% (w/v) TCA. The reaction was incubated at 80℃ for 30 min on a hot plate in a fume-hood and then stopped in a water bath. The samples were centrifuged again at 13,500 × rpm for 5 min. Absorbance was measured at 532 and 600 nm using a 96-well plate reader. The MDA concentration was calculated using the Beer-Lambert equation: $nmol\ MDA / g\ DW = (DA_{corrected} \times df \times x \times 1000) / e \times b \times y$

where:

$DA_{corrected}$: Absorbance at 532 nm - absorbance at 600 nm

b: Light path length (in cm)

ε (epsilon): Extinction coefficient for the MDA-TBA complex (155 mM^{-1} cm^{-1})

df: Dilution factor

x: Volume of TCA (µL) extract used for reaction

y: Fresh weight (FW) of the tissue sample 1000 = conversion factor

3.2.5.2 Proline content

Proline was measured according to the protocol of Campobenedetto et al. (2021). Approximately 0.1 g of the ground leaf samples were homogenized in 1000 µL of 3% (w/v) sulfosalicylic acid. The samples were centrifuged for 5 min at room temperature at 13,000 x rpm. Approximately 200 µL of the supernatant was added to 500 µL of the reaction mixture (100 µL of the 3% sulfosalicylic acid + 200 µL glacial acetic acid + 200 µL acidic ninhydrin). This was repeated for the preparation of the concentration of the standard solution. The prepared solutions in 2 ml tubes were incubated at 96°C for a 1 h. About 1 mL of toluene was added to the reaction mixture, and the polar and non-polar phase were allowed to separate for 5 min. Approximately 200µL of the top phase was removed and transferred to a 96 well plate. The absorbance was measured at 520 nm. The proline concentration was determined using a standard curve basis and calculated on fresh weight.

3.2.5.3 Antioxidant activity

The measurement of the antioxidant capacity of the extracts was performed with assays based on electron transfer (Et) (Škrovánková, Mišurcová & Machů, 2012). These methods included DPPH (2,2-diphenyl1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power).

3.2.5.3.1 Determination of antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method

The DPPH assay was carried out according to Dudonne et al. (2009). Approximately 0.1 g of the leaf samples were ground with 1000 µL of methanol and placed in a shaker for 24 h at room temperature. The samples were then filtered through a Whatman qualitative filter paper, Grade 1. A 200 µL aliquot of the methanol extract was added to 400 µL of dilute DPPH (prepared by mixing 1 mL extract with 400 µL DPPH, diluted to a final concentration of $1x10^{-4}$ M with methanol) in a 96-well plate. The absorbance of DPPH was measured at 517 nm, and its concentration was determined using a molar extinction coefficient of 11,200 M^{-1} cm^{-1}. The concentration was calculated using the Beer– Lambert's equation:

DPPH scavenged (µmol/g FW): $\mu mol / g\ DW = (DA_{corrected} \times df \times x \times 1000) / e \times b \times y$

grams)

where:

Absorbance difference: $Absorbance_{blank}$ - $absorbance_{sample}$ at 517 nm
b = light path length (0.56 cm for 200 µL).

ε (epsilon): = millimolar extinction coefficient (11,200 M^{-1} cm^{-1})

df = 3.5 (i.e., dilution factor from 1 mL extract + 400µL Dilute DPPH to a concentration of $1x10^{-4}$M with methanol)

x (mL) = methanol used for extraction (1 mL)

y (g) = FW of tissue used for extraction (100 mg = 0.1 g)

ε (epsilon): millimolar extinction coefficient

3.2.5.3.2 Ferric reducing antioxidant power (FRAP) method

The FRAP assay was carried out according to Dudonne et al. (2009), with similarities to the DPPH method (Section 3.1.6.1). Approximately 0.1 g of leaf samples were macerated in 1000 µL of methanol and incubated on a shaker at room temperature for 24 h. The samples were then filtered through Whatman Grade 1 qualitative filter paper.

A 200 µL aliquot of the methanol extract was added to 400 µL of the FRAP reagent (10:1:1 mixture of 300 mM sodium acetate buffer solution (pH 3.6), 10 mM TPTZ, and 20 mM $FeCl_3 \cdot 6H_2O$). The reaction mixture was aliquoted into wells of a UV-96 well plate and incubated for 5 minutes at 37℃ in the dark. The absorbance was measured at 593 nm, using a molar extinction coefficient of 21,140 M^{-1} cm^{-1}. The concentration was calculated using the Beer– Lambert's equation:

FRAP (µmol/g FW) = $\mu mol / g\ DW = (DA_{corrected} \times df \times x \times 1000) / e \times b \times y$

where:

Absorbance difference: Absorbanceblank - absorbancesample at 593 nm

b: light path length (0.56 cm for 200 µL). Confirm with your plate reader.

ε (epsilon): millimolar extinction coefficient (21,140 $^{-1}$ cm^{-1})

df: 3.5 (i.e., dilution factor from 1 mL extract + 400 µL 300 mM sodium acetate buffer solution (pH 3.6) + 10 mM TPZT+ 20 mM $FeCl_3 \bullet 6H_2O$ solution)

x (mL): methanol used for extraction (1 mL)

y: FW of tissue used for extraction (100 mg = 0.1 g)

3.3 Results and Discussion

3.3.1 Physiological and morphological analyses

3.3.1.1 Morphological measurements

Morphological measurements of the tomato plants were taken for the four treatment groups following the heat treatments, as outlined in the methodology (section 3.2.2). To assess the effect *of E. maxima* on tomato plants, both shoot and root lengths were measured using a ruler, Figure 3.3 shows the root measurements for all four treatment groups.

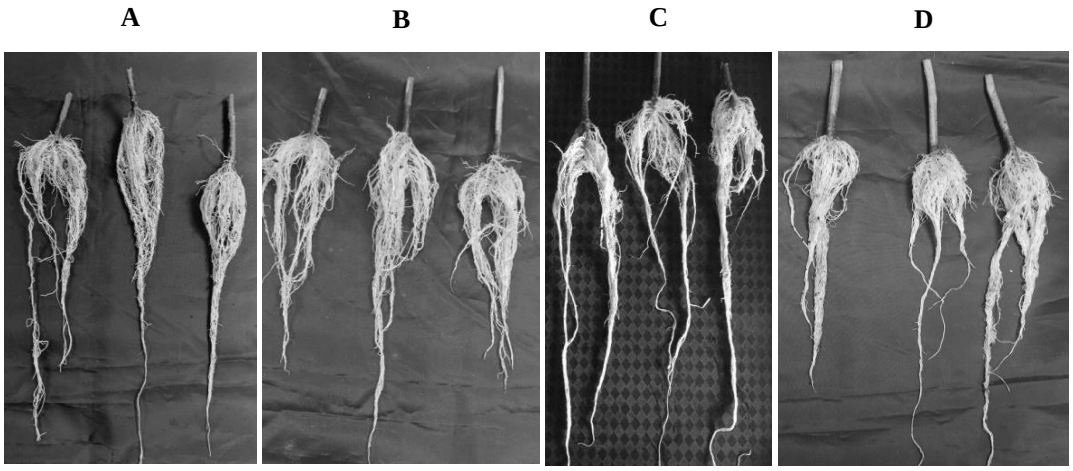

Figure 3.3: Tomato plants after HS and control treatments. Sectioned roots showing A) control [C], B) biostimulant control [B], C) heat shock [HS], and D) biostimulant and heat shock [BH].

No distinct differences between the roots of treatment groups were observed including the treatment groups after the HS treatment (Figure 3.3). This may be because both the water control and *E. maxima* derived biostimulant were applied as a foliar spray to the leaves and not as a soil drench. In addition, the treatment duration may have been too short, as the foliar application was limited to two consecutive days, which might not have been sufficient to induce any noticeable changes in the root structure with or without HS stress.

The morphological characteristics of the tomato shoots were also examined between the four treatment groups (Figure 3.4).

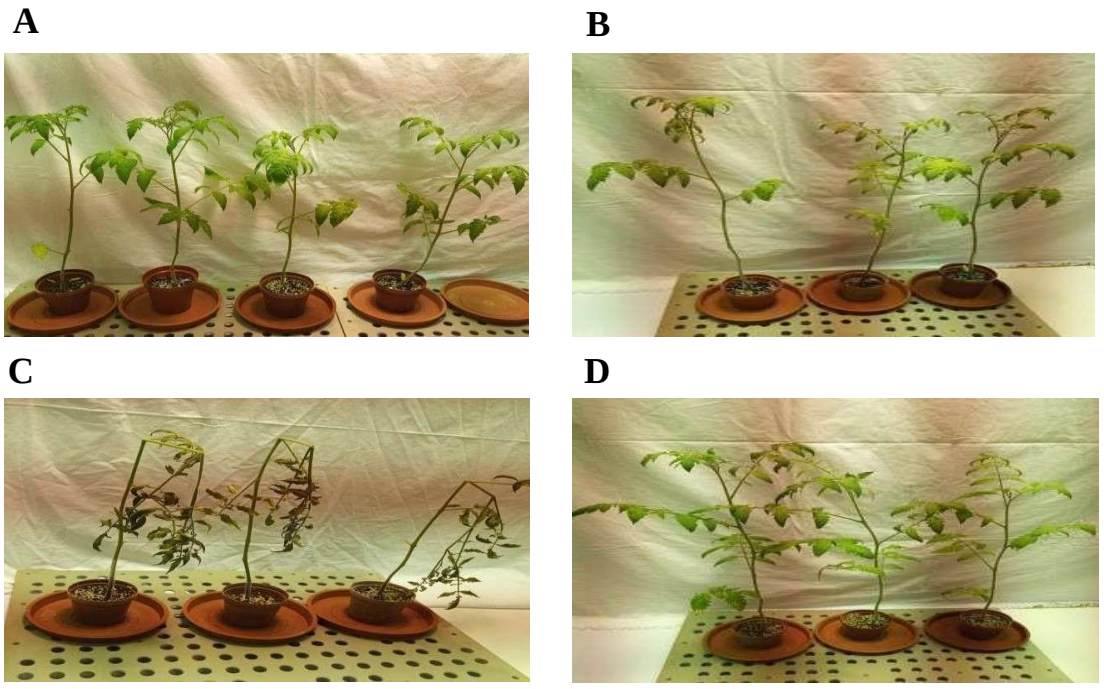

Figure 3.4: Tomato plants after the HS and control treatments showing A) control [C], B) biostimulant control [B], C) heat shock [HS], and D) biostimulant and heat shock [BH].

There were no observable morphological changes in the tomato leaves between the C and B treatments before HS stress treatments (Figure 3.4 A and B) as both treatment groups were grown under optimal controlled conditions. In contrast, the H treatment group displayed distinct morphological changes after HS stress treatment (Figure 3.4 C). There was distinct leaf drooping, leaf rolling or curling and bending of the main stem. What is not clearly visible in the image but was observed was chlorosis on the majority of the leaves due to the heat shock stress. Higher temperatures often promote leaf curling in plants as a mechanism to minimize leaf surface area thereby conserving moisture due to the heat stress. The observed chlorosis may be due to the over accumulation of ROS which causes oxidative damage to the chloroplast, mitochondria, and other organelles, contributing to leaf senescence and cell death (Parent et al., 2008).

Despite the negative effects of the HS stress treatments, the BH treatment had none of these morphological characteristics associated with the H treatment (Figure 3.4 D). The leaves maintained their green color, shape, and remained upright after the heat shock stress treatment suggesting that prior application of the *E. maxima*-derived biostimulant enhanced thermotolerance in tomato shoots for subsequent heat stress events.

These observations are consistent with a previous study which showed that prior foliar

application of an A. *nodosum*-derived SE biostimulant to turf grass (*Agrostis stolonifera*), which was subjected to both heat and drought stress resulted in improved turf quality, leaf color ratings and increased chlorophyll concentrations (Zhang et al., 2023).

3.3.1.2 Electrolyte leakage

Electrolyte leakage in plants is the loss of ions, such as potassium (K^+), from cells into the surrounding tissue, leading to compromised cell membrane integrity and programmed cell death. This loss of ions, as measured by electrolyte leakage, is commonly observed under various abiotic stresses including HS (Demidchik et al., 2014). In this study, it was observed that the electrolyte leakage for the H treatments was statistically significantly higher than the C and B treatments (Figure 3.5). Though the B treatment was slightly higher than the C treatment, it was not statistically significant. What was also further noted was that the BH treatment was significantly lower than the H treatment.

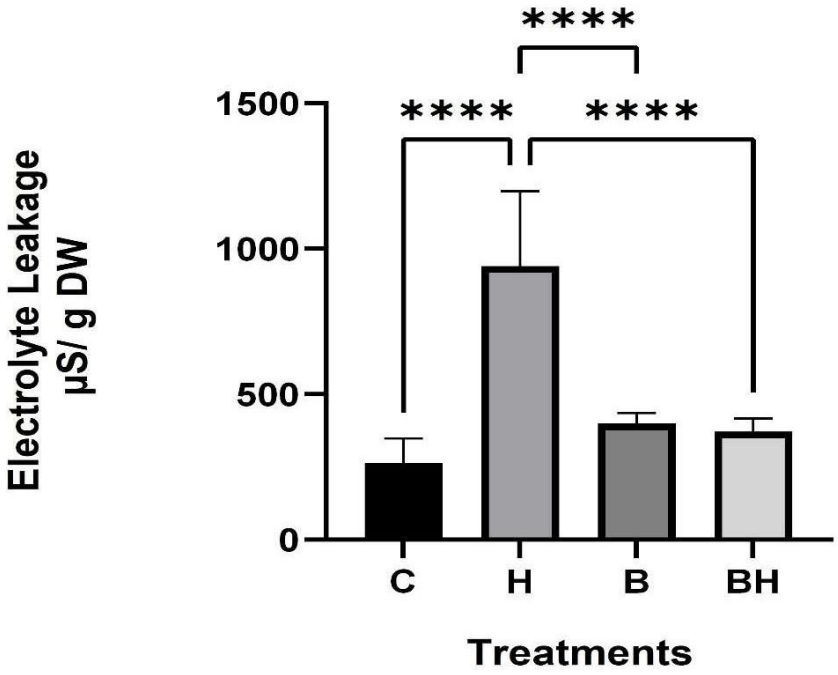

Figure 3.5: Electrolyte leakage measurements of the tomato leaf (middle section) for the following treatment groups: control [C], heat shock [H], biostimulant control [B], and biostimulant heat shock [BH], were performed on three biological replicates each [n=3]. Error bars indicate standard error of the mean with the level of significance indicated by the asterisk as determined by the one-way ANOVA test (***, $p \leq 0.001$) and Tukey-HSD post-hoc test.

Heat stress increase ROS levels (Allakhverdiev et al., 2008), which damages the plasma membrane leading to increased membrane permeability with the resulting efflux of electrolytes from the cell initiating programmed cell death (Demidchik et al., 2014 & Sharma et al.,

2019). The very high increase in electrolyte leakage observed for the H treatment could therefore, be attributed to ROS damage of the plasma membrane.

SE-derived biostimulants act as a priming agent in initiating stress memory to help plants withstand and/or improve tolerance to subsequent stresses such as heat shock (Nephali et al., 2020). The SE-derived biostimulants modulate central metabolic pathways such as photosynthesis and activate defence pathways to increase ROS scavenging (Shukla et al., 2019). It was observed that *E. maxima* treated plants subject to HS (BH) had minimal damage to their plasma membrane as seen by the low electrolyte leakage and when compared to the C and B treatments (Figure 3.5). This suggests that *E. maxima*-derived biostimulant primed the tomato plants for the subsequent heat shock stress resulting in enhanced heat tolerance during the heat shock treatments.

3.3.1.3 Chlorophyll fluorescence and photosynthetic pigment content measurements

Chlorophyll parameters, especially chlorophyll fluorescence and quantification of photosynthetic pigments are common, and non-invasive methods used to assess the photosynthetic performance of plants, providing insights into their health to different environmental conditions. Photosystem II (PSII) is a protein complex embedded in the thylakoid membrane of plant chloroplasts, which is responsible for the initial step of photosynthesis (Shen, 2015). The chlorophyll fluorescence parameter Fv/Fm reflects the maximum quantum efficiency of photosystem II (PSII) and is widely used for early stress detection in plants. The photosynthetic pigment assays provide information on the chlorophyll pigment content and indicate potential damage or adaptation in response to stress (Sharma et al., 2015).

Under light-adapted (ΔF/Fm') conditions (Figure 3.6A), the C treatment exhibited the highest Fv/Fm value, reflecting efficient photosynthetic performance of PSII.

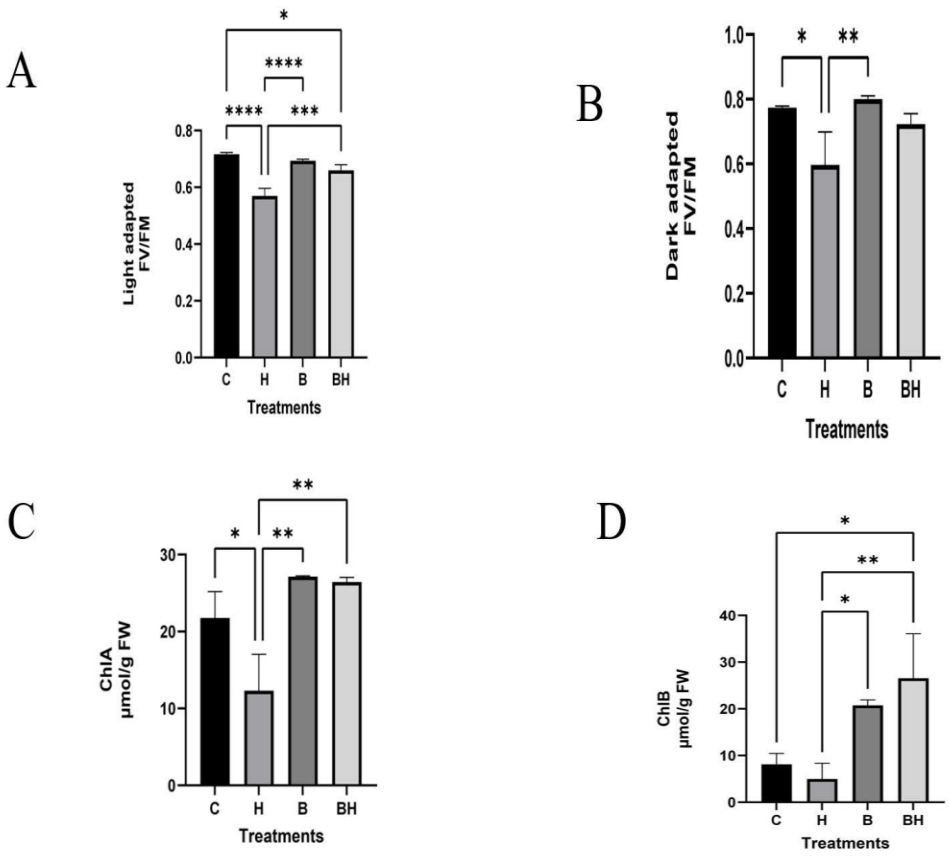

Figure 3.6: Chlorophyll measurements showing chlorophyll fluorescence of [A] light adapted (ΔF/Fm'), [B] dark-adapted (Fv/Fm), and photosynthetic pigments [C] chlA and [D] chlB from tomato leaves from the four treatment groups, control [C], heat shock [H], biostimulant control [B], and biostimulant heat shock [BH]. The experiments were performed on three biological replicates each [n=3]. Error bars indicate standard error of the mean with the level of significance indicated by the asterisk as determined by the one-way ANOVA test (***, $p \leq 0.001$) and Tukey-HSD post-hoc test.

The H treatment had the lowest statistically significant Fv/Fm value when compared to the C and B treatments indicating significant impairment of PSII efficiency. This is most likely due to the plasma membrane being compromised by the heat shock as confirmed with the increase in electrolyte leakage (Figure 3.5, section 3.2.1.2). PSII is extremely susceptible to environmental stress, especially HS and the resultant oxidative stress (Aro et al., 1993). These abiotic stresses inhibit the repair of the photodamage to PSII by inhibiting the PSII protein synthesis, leading to a decrease in photosynthetic efficiency (Takahashi & Murata, 2008).

There was a slight reduction in Fv/Fm of the B treatment compared to the C treatment, but it was not statistically significant. There was also no significant difference between the B and

BH treatments. However, the BH treatment displayed statistically significantly higher photosynthetic efficiency than the H treatment, suggesting that priming with the *E. maxima*-derived biostimulant helps mitigate the negative effects of heat shock stress on PSII performance.

A similar trend was observed for the dark-adapted conditions (ΔF/Fm) (Figure 3.6 B) when compared to the results obtained for light-adapted conditions, The C treatment group maintained an Fv/Fm value similar to what was observed for the light adapted conditions indicating fully functional PSII. The H treatment had a statistically significant reduction when compared to both the C and B treatments indicating impairment of the PSII. Notably, the BH group restored Fv/Fm levels almost close to those of the C group, highlighting the protective effects of the biostimulant against heat-induced damage which were seen like the light adapted results.

The chlorophyll pigments chlA and chlB were negatively impacted by HS stress (Figure 3.6 C & D). For chlA there was a statistically significant decrease in the H treatment when compared to the C treatment but not in the case for chlB. However, the ratio of chlA to chlB was greater than three in the C treatment which is considered an indicator of optimal photosynthesis and plant health (Ngcobo et al., 2024), due to the positive correlation with the ratio of PSII cores to the light-harvesting chlorophyll–protein complex (Apostolova et al., 2006). Both chlorophyll pigments increased in the B treatment compared to the C and H treatments (Figure 3.6 C&D). Importantly, both chlA and chlB pigments were statistically significantly higher in the BH treatment when compared to the H treatment indicating that prior application of *E. maxima*- derived biostimulant reduced the negative impact of heat stress shock on chlorophyll pigment content.

These results are similar to the Makonya et al. (2025) study who found that a biostimulant derived from *Ascophyllum nodosum* extract helped raspberries mitigate the effect of high temperature stress. They showed that the kelp-derived biostimulant helped maintain high chlorophyll fluorescence (Fv/Fm) and photosynthesis under high temperature stress treatments providing thermotolerance to the biostimulant treated plants. Overall, H treatment results in the reduction of chlorophyll pigments and the greatest disruption to PSII efficiency under both light and dark adaptation compared to the C and B treatments. The BH group demonstrated a protective role, mitigating the HS stress and maintaining photosynthetic efficiency near to the control levels.

3.3.2 Antioxidant assays

As mentioned previously (Chapter 2, section 2.2) high temperature stress can result in the overproduction of ROS which leads to oxidative stress related damage to biomolecules, such as proteins, pigments, carbohydrates, lipids and DNA and eventual cell death. The plant has various antioxidant systems that aim to either maintain ROS homeostasis and/or increase antioxidant capacity in response to excess ROS due to abiotic stress (Das & Roychoudhury, 2014; Mittler, 2017). In order to assess antioxidant capacity in tomato leaf tissue for the different treatment groups the following assays were performed; lipid peroxidation, proline content, 2,2-diphenylpicrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP).

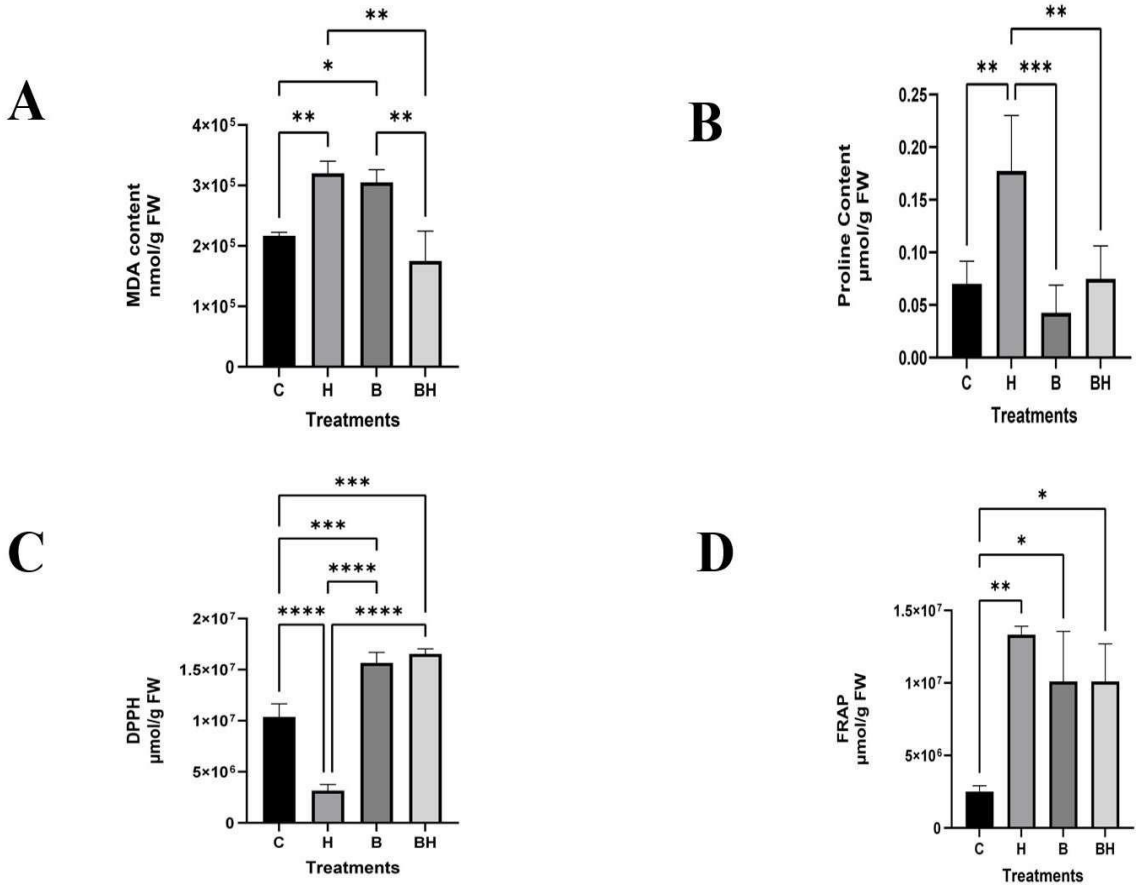

Figure 3.7: Antioxidant assays showing the results for: [A] Lipid peroxidation (MDA), [B] Proline, [C] 2,2-diphenylpicrylhydrazyl (DPPH), and [D] ferric reducing antioxidant power (FRAP). All materials were obtained from tomato leaves in the four treatment groups, control (C), heat shock (H), biostimulant control (B), and biostimulant heat shock (BH). The experiments were performed on three biological replicates each [n=3]. Error bars indicate standard error of the mean with the level of significance indicated by the asterisk as determined by the one-way ANOVA test (***, $p \leq 0.001$) and Tukey-HSD post-hoc test.

3.3.1.1 Lipid peroxidation

The lipid peroxidation assay is used as a key indicator of oxidative stress and cell damage in plants caused by environmental stressors such as HS. The breakdown of lipids in cell membranes results in the accumulation of ROS within the plant and the release of the toxic by-product of lipid peroxidation malondialdehyde (MDA) which is measured by the assay (Awasthi et al., 2018). We observed that the H treatment significantly increased the MDA content compared to the C treatment (Figure 3.7 A), indicating elevated lipid peroxidation and oxidative stress damage caused by the heat treatments.

The B treatment also resulted in higher MDA levels compared to the C treatment suggesting an increase in ROS and oxidative processes. However, the BH treatment statistically significantly reduced the MDA content relative to the H and B treatments. The increase in MDA levels under HS stress highlights the extent of oxidative damage caused by stress, as lipid peroxidation is a marker of cellular injury under such conditions (Al-Zahrani, Alharby & Fahad, 2022). The elevated MDA levels observed in plants treated with the *E. maxima*-derived biostimulant alone (B treatment) may suggest that the *E. maxima*-derived SE induces mild oxidative stress, potentially as part of a stress priming effect. This hypothesis is supported by the significant reduction in MDA content in the BH treatment, indicating that the *E. maxima*-derived biostimulant enhanced the tomato plant's thermotolerance to prepare the plant for the increased oxidative damage as a result of the HS stress treatment.

This result is similar to Anjos Neto. (2020) who evaluated the effects of priming spinach seeds with *A. nodosum* SE on germination and growth under heat stress. It was observed that primed seedlings had lower contents of MDA under heat stress than the control spinach seeds. The lipid peroxidation assay results suggest that the *E. maxima* primes the tomato plant defence systems, enabling a more efficient response to heat shock stress to mitigate the negative impact of lipid peroxidation.

3.3.1.2 Proline content

Proline is an amino acid that accumulates under adverse stressful conditions such as heat stress and plays a role in plant development and stress tolerance (Hayat et al., 2012)(Dutta et al., 2018). Proline functions as an osmoprotectant, stabilizing proteins and cell membranes and alleviates and protect plant cells from the deleterious effects of oxidative stress induced by HS by acting as a ROS scavenger (Hayat et al., 2012).

We observed a statistically significant increase in proline content in H treatment compared to

the C, B and BH treatments (Figure 3.7 B), which indicates oxidative stress during the HS stress treatment. Notably the BH treatment had proline content similar to the C treatment but statistically significantly reduced compared to the H treatment indicating that prior application of *E. maxima*-derived biostimulant modulated the stress response to subsequent heat shock stress treatment.

There was a slight reduction in proline content in the B treatment compared to the C treatment, but it was not significant. Studies have found that proline content is typically reduced in plants treated with SE biostimulants. One of the reasons given is that the composition and/or formulation of the SE biostimulant could contribute to lowering proline content and the need for its protective role. In a drought stress study on tomatoes, Campobenedetto. (2021), tested the effect of SE biostimulant extract consisting of *Ascophyllum laminaria digitata* and yeast extract. It was found that though proline content was lower in the SE biostimulant treated drought stress tomatoes, it also correlated with lower activity of ROS scavenger enzymes compared to untreated plants. The authors suggested that the SE biostimulant contains antioxidant molecules, such as flavonoids and flavanols, which are able to contribute to ROS scavenging and therefore indirectly reduce proline's osmoprotective role and by extension its accumulation in response to stress. Furthermore, *E. maxima*-derived biostimulant has been found to contain compounds that enhance plant growth, support root development, stimulate cell division, elongate stems, and bolster stress tolerance (Lefi et al., 2023). Similarly, these compounds could either mimic proline's role in protecting cell membranes and stabilizing proteins or reduce the amount of osmoprotection contributed by proline to the cell, also indirectly reducing its accumulation. Moreover, it was observed in a drought stress study that wheat seedlings that were primed with *Ulva linza* SE-derived biostimulant also had reduced proline content when compared to control seedlings (Hamouda, Saad-Allah & Gad, 2022). It was noted that the decrease in proline concentration was linked to the increase in total soluble proteins, which could indicate that proline was integrated into protein synthesis.

These previous studies suggest that SE biostimulants may improve ROS homeostasis, directly through its composition, and/or activate alternative ROS scavenging mechanisms, mitigating stress severity and reducing the need for proline as an osmoprotectant (Carvalho et al., 2018).

3.3.1.3 DPPH measurements

DPPH is a stable free radical that absorbs light at a specific wavelength of 517 nm, giving it a deep purple color. When antioxidants are present, they react with the DPPH radical, reducing

it to a non-radical form, which results in a loss of the purple color and change to a pale-yellow color (Silva et al., 2024).

The H treatment resulted in significantly lower DPPH levels when compared to the C treatment, indicating oxidative stress leading to the accumulation of ROS and therefore suggesting increased antioxidant activity to mitigate ROS damage (Figure 3.7 C). The B treatments had significantly higher DPPH levels than the C treatment which indicates an enhanced antioxidant systems and modulated ROS homeostasis. However, the combined BH treatment displayed the highest DPPH levels when compared to the C and H treatments, with both being statistically significant.

As previously discussed, when assessing chlorophyll, antioxidant, MDA and proline content (Figure 3.7), HS stress disrupts cellular processes, specifically in the chloroplasts and mitochondria, leading to the accumulation of ROS. Excess ROS further leads to oxidative damage to lipids, proteins, and DNA (Liu & Lin, 2020; Que et al., 2018). In the event of elevated temperatures, plants activate enzymatic antioxidants such as superoxide dismutase, catalase, peroxidase and non-enzymatic antioxidants such as flavonoids and phenolics (Hasanuzzaman et al., 2020). SE biostimulant application as a priming agent enhances plant resilience to heat stress by improving antioxidant capacity (Raja & Vidya, 2023). In addition, the presence of various compounds in *E. maxima* facilitates the process of promoting biosynthesis of antioxidative secondary metabolites, which leads to an improved defence mechanism against abiotic stress (Boutahiri et al., 2024), by improving ROS scavenging, and activating antioxidant systems and increasing the production of ROS-scavenging enzymes, ultimately leading to improved plant heath and productivity (Gatti et al., 2024). This suggests that *E. maxima*-derived biostimulant mitigates negative effects of HS stress by directly or indirectly enhancing antioxidant capacity.

3.3.1.4 Ferric reducing antioxidant power (FRAP)

The ferric reducing power (FRAP) assay is a technique used to analyze the total antioxidant capacity of a sample by determining its ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in an acidic medium, this then results in a blue- colored ferrous-probe complex when measured spectrophotometrically (Payne et al., 2013).

High temperature stress of plants leads to increased cellular production of ROS, which can cause oxidative damage to cellular components. To minimize these harmful effects, plants activate their antioxidant defence systems, which comprise of both enzymatic and non-

enzymatic antioxidants (Hasanuzzaman et al., 2020). This increased antioxidant capacity can assist in thermotolerance depending on the nature and duration of the heat stress and the robustness of the plant antioxidant response.

The H treatment statistically significantly increased FRAP levels compared to the C treatment, indicating increased antioxidant capacity in response to oxidative stress caused by the HS treatment (Figure 3.7 D). The B treatment resulted in higher FRAP levels compared to the C treatment indicating that the application of *E. maxima*-derived biostimulant enhanced antioxidant capacity independent of any abiotic stress and suggesting priming of stress-adaption for plant resilience to future stress events.

Both the B and BH treatments had similar FRAP levels and both had lower FRAP levels than the H treatment but not significantly so (Figure 3.7 D). The BH treatment also had increased FRAP levels compared to the C treatment and given that it was also similar to the B treatment, it suggests that prior application of the *E. maxima*-derived biostimulant could have had already activated antioxidant systems to better response and tolerate the later heat shock stress.

3.4 Conclusions

This chapter aimed to investigate the effect of *E. maxima*-derived biostimulant (Afrikelp^{TM}) on tomato plants subjected to heat shock (HS) treatments, with a focus on morphological, physiological, and biochemical changes.

The results from the morphological experiments indicate that root growth was not significantly influenced by the treatment most likely due to the foliar application method on the shoots and the duration of the foliar treatment. In contrast, more noticeable differences were observed in shoot growth, particularly between the heat shock (H) and biostimulant + heat shock (BH) treatment groups. The H treatment exhibited signs of oxidative stress, such as leaf curling, stem bending, and colour change, which are typical indicators of heat-induced damage. However, the BH treatment maintained better structural integrity, with preserved leaf pigmentation and overall plant form.

Electrolyte leakage assays confirmed plasma membrane damage for the H treated plants. In contrast tomato plants treated with *E. maxima*-derived biostimulant and subjected to heat shock (BH) had minimal damage to their membrane which suggests priming of the tomato plants by the biostimulant for enhanced thermotolerance for subsequent heat shock stress.

Photosynthetic (PSII) efficiency was negatively affected in the H treatments, however the BH

treatment restored photosynthetic efficiency close to levels of the C treatment. Similarly, the chlorophyll pigments chlA and chlB were reduced in response to heat shock stress and increased in both the B and BH treatments compared to the control plants. This demonstrates that prior application of *E. maxima*-derived biostimulant reduced the negative impact of heat stress shock on photosynthesis to maintain plant performance.

The antioxidant assays (MDA, proline, DPPH and FRAP) support the idea that prior application of *E. maxima*-derived biostimulant directly or indirectly enhances antioxidant capacity and acts as a priming agent. Lipid peroxidation, as measured with MDA, was highest in H treatments and interestingly, MDA levels were high in the biostimulant only (B) treatment. This may suggest that the *E. maxima*-derived SE induces mild oxidative stress, potentially as part of a stress priming effect. This hypothesis is supported by the significant reduction in MDA content in the BH treatment, indicating that the *E. maxima*-derived biostimulant primed the tomato plants for the HS stress. The proline content, as expected, was high in the H treatment but significantly lower in the B and BH treatments. Previous studies have suggested that SE biostimulants lower proline content either due to antioxidants that occur in the SE biostimulants and/or they enhance osmoprotection mechanisms in the plant thereby reducing the need for high levels of proline for stress protection. The DPPH assay indicated an increase in antioxidant activity for the H treatment as observed by the lower DPPH levels compared to the control. The B treatments had higher DPPH levels to the control which indicates that antioxidant systems are modulating ROS homeostasis for subsequent stress. Notably, the BH treatment displayed the highest DPPH when compared to the C, B and H treatments indicating that the *E. maxima*-derived biostimulant enhanced ROS scavenging of antioxidant systems, directly or indirectly, to mitigate the HS stress. This result was also supported by the FRAP assay which measures antioxidant capacity where FRAP levels were high in the H treatments compared to the control indicating increased antioxidant capacity. The B and BH treatments also resulted in higher FRAP levels compared to the C treatment indicating that the application of *E. maxima*-derived biostimulant activated antioxidant systems prior to the HS stress. This would allow the conditioned tomato plants to better respond and tolerate the later heat shock stress.

In conclusion, we showed that the *E. maxima*-derived biostimulant acts as a priming agent to enhance and protect photosynthesis while improving thermotolerance to HS stress by directly and/or indirectly enhancing antioxidant capacity in the plants.

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Chapter 4

A metabolomic approach to investigating the response of tomato to *E. maxima* treatment after heat shock exposure

4.1 Introduction

In general, plants respond to high temperatures through a combination of short-term avoidance mechanisms including; leaf orientation, transpiration, cooling, long-term adaptations such as membrane lipid composition and early maturation, as well as by activating molecular pathways for heat stress protection (Bita & Gerats, 2013). Metabolomics has become an emerging field for the past four decades (Fernie et al., 2004) and has enabled a better understanding of metabolic systems involved in heat stress and heat tolerance in plants. Heat shock (HS) stress is a critical abiotic stress factor globally and affects the growth and productivity of plants and crops. The coupling of modern breeding technologies with metabolomics has significantly aided investigations into crop HS tolerance (Raza, 2022). Several studies have demonstrated the crucial roles of amino acids, sugars, and secondary metabolites in modulating a HS response. The identification of HS metabolic biomarkers could be used as a targeted and fast diagnostic tool to assist in identifying the germplasm of plants, with an improved HS tolerance (Thomason et al., 2018).

Of the several approaches to characterizing the plant metabolome, gas chromatography-mass spectrometry (GC-MS) is ideal for identifying and quantifying small molecular metabolites, such as acids, alcohols, hydroxyl acids, amino acids, sugars, fatty acids, and sterols. One of the major advantages of GC-MS is the availability of both public and commercial spectral libraries, facilitating the identification of compounds by matching their mass spectra to reference data, although distinguishing structural isomers often requires additional analytical techniques (Halket et al., 2005).

The rationale behind this chapter was to use a GC-MS metabolomics approach to investigate the specific amino acids, sugars, sugar alcohols, and/or organic acids that are potentially primed in tomato plants subjected to HS and prior treatment with *E. maxima*-derived biostimulant. Investigating the abundance or presence of these metabolites in conjunction with the data from the previous chapters may further the understanding of the biological processes involved in the priming mechanism of *E. maxima* in tomato plants when subjected to heat shock.

4.2 Materials and Methods

4.2.1 Plant material, heat treatments, and sampling

Tomato plants were maintained and heat treated as described in Chapter 3 (section 3.2.1 and 3.2.2).

4.2.2 Extraction and derivatization

The extraction and derivatization protocol was conducted as previously described by Lisec et al. (2006) and Valledor et al. (2014) with the following modifications: 300 mg of the frozen fresh weight (FW) leaf tissue were dried overnight in the freeze dyer. Once the sample weight stopped decreasing, the sample dry weight was recorded, and the relative water content calculated using this formular:

$$\textbf{RWC (\%)} = (DW/FW) \times 100$$

Approximately 1 mL of the methyl tert-butyl ether (MTBE) and methanol extraction mixture containing mg/mL ribitol (internal standard) was added to 2 mL Eppendorf screw lid tubes containing the 20 mg ground dry tissue. The samples were vortexed at 4℃ in an orbital shaker for 30 min at 12,000 × rpm. The samples were then incubated for another 15 min in a sonication bath consisting of ice water and thereafter centrifuged at room temperature for 5 min at 14,000 × rpm. Aliquots of 800 µL, taken from the green upper phase, were transferred to a new 1.5 mL Eppendorf tube. A volume of 800 µL of methanol water (1:3) was added to the samples and vortexed. The samples were then centrifuged for 3 min at 14,000 × rpm. The same procedure was repeated for the blank samples. A volume of 600 µL of the lower phase was transferred to a new 1.5 mL Eppendorf tube. The samples were then dried using a SpeedVac which is primarily used for concentrating or drying liquid plant extracts. After drying the samples were then stored at -80℃.

Derivatization was carried out the next day by addition of the methoxyamination (MSTFA) reagent to prepare the samples for GC-MS analysis. The samples were removed from the -80℃ freezer and placed in a SpeedVac to dry for approximately 20-30 min. A volume of 40 µL of freshly prepared pyridine was added to the samples in the fume hood. The samples were then placed in an orbital shaker at 37℃ for 2h at 1,000 × rpm. The aliquots were then spun for 10s before the addition of 70 µl MSTFA. Samples were incubated at 37℃ for 30 minutes at 1,000 × rpm.

4.2.3 Sample processing for GC-MS analysis

The derivatized samples were centrifuged for 2 min at 14,000 × rpm and thereafter, approximately 100 µL of the aliquots were transferred to glass vials suitable for GC-MS analysis. An in-house library of standards used for analysis was previously prepared by Dr Shandry Tsebele (Plant Stress Lab, University of Cape Town). All the compounds were identified and verified based on the fragmentation pattern, base peak m/z and retention time of

each standard.

4.2.4 GC-MS metabolite profiling of sugars, amino acids, sugar alcohols, organic acids and phytohormones

After derivatization, the samples were run on an Agilent 7890A gas chromatography system equipped with an Aglient autosampler 7693 and Agilent 7000C Triple Quadrupole mass spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). About 1 µL of the sample was injected into the GC (split 19:1), with the oven program starting with the initial temperature from 80℃ to 320℃. The injector, transfer line and ion source temperatures were as follows 240℃, 280℃ and 230℃, respectively. The filament voltage was 70 eV. The chromatogram then scanned for 6 min for ion from 70 to 500 m/z, 4.8 scan/sec.

4.2.5 Peak identification

The MassHunter B.09 Quantification software (Agilent, Germany) was used to identify and quantify metabolites by comparing the spectra. The identification and quantification of the metabolites was based on the NIST14 MS library (National Institute of Standards and Technology, Gaithersburg, MD, USA), the Fiehn library as well as comparing to the retention times developed by Dr Tsebele (pers comm.). The metabolites obtained were then used to establish a master peak list. The output was generated in Excel format, including columns for the various samples and rows for the detected metabolites. The metabolites were quantified as peak areas.

4.2.6 Data pre-processing

The raw datasets were interrogated by removing all contaminants based on sample comparison, and metabolites with incorrect ionization values were also removed based on the retention times indicated by the standards. The polished raw data was then further pre-processed by normalization with the internal standard ribitol peak area and sample dry weight to reduce the bias in samples. The final data was then exported in the form of a comma-separated values (CSV) file and upload to Metaboanalyst6.0 (https://www.metaboanalyst.ca) for statistical analysis.

4.2.7 Identification of significant metabolites

The ANOVA Fishers LSD post-hoc analysis method with $p < 0.05$ was performed on partial least square analysis (PLS-DA) on metabolites identified and cross validated. The heatmap

hierarchical clusters was based on the difference in the significant metabolites found in the plants subjected to the 4 different treatments, which was used to further validate the PLS-DA clusters and ANOVA results (Zhao et al., 2014). The heatmap was generated from the normalized data and hierarchical clusters represented using Euclidean distance, a clustering method of the four groups treated.

4.2.8 Fold change analysis

The fold change analysis was used on the targeted metabolites to compare the log_2 transformed ratio between each metabolite in each of the four treatment groups (Xia et al., 2009). The fold change analysis was performed to identify the differentially abundant metabolites relative to the four groups of treatments across the plant tissues.

4.2.9 Pathway analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG)

To interpret the results generated from fold change analysis and ANOVA, a pathway analysis found within MetaboAnalyst 6.0 was used to verify and visualize the functional interpretation of the differentially abundant metabolites.

Metabolic pathway analysis consisted of graphs to visualize the data where circles coloured in shades of red/orange indicate the corresponding metabolites that are upregulated or present at higher concentrations compared to a control or reference. The deeper or more intense the red, the greater the degree of the upregulation, including a lower p-value indicating a more statistically significant enrichment (Tsouka & Masoodi, 2023).

Only well-annotated compounds based on pathway libraries were mapped. Mapping was done with the KEGG database for *Arabidopsis thaliana* to identify the significant pathways of the upregulated metabolites and associations between pathways to represent pathways shared across the groups.

4.3 Results and Discussion

4.3.1 Variation of metabolites across the heat treatment stages

It was necessary to first determine whether there was a difference in the metabolites during the HS treatment stages in tomatoes by performing the partial least square analysis (PLS-DA) (Figure 3.9). The PLS-DA, a statistical method used for classifying data in high-dimensional datasets, particularly when the number of variables significantly exceeds the number of samples, making it ideal for identifying key features that differentiate between groups in complex data like metabolomics was used (Lee et al., 2018).

This was performed to visualize the separation of metabolites across the individual tissue types and the four treatment groups. The score plots were conducted to analyze the variance of the various metabolites including amino acids, sugars, sugars, sugar alcohols, and organic acids.

In the PLS-DA score plots (Figure 4.1), the B treatment versus C treatment, H treatment versus C treatment, and BH treatment versus C treatment showed distinct separation. The first two components explain varying degrees of covariance between predictors and class labels. For the B treatment versus C treatment, the component 1 explained 26.5% and component 2 explained 23.7% of the variation, suggesting moderate class separation. In the H treatment versus C treatment, component 1 explained 32.9% and component 2 explained 30.8%, indicating a stronger separation between these groups. For BH treatment versus C treatment, component 1 explained 58.4% and component 2 14.3%, showing a prominent separation along the first component. These results suggest that the metabolites in tomatoes leaf material varried significantly between the four treatment groups.

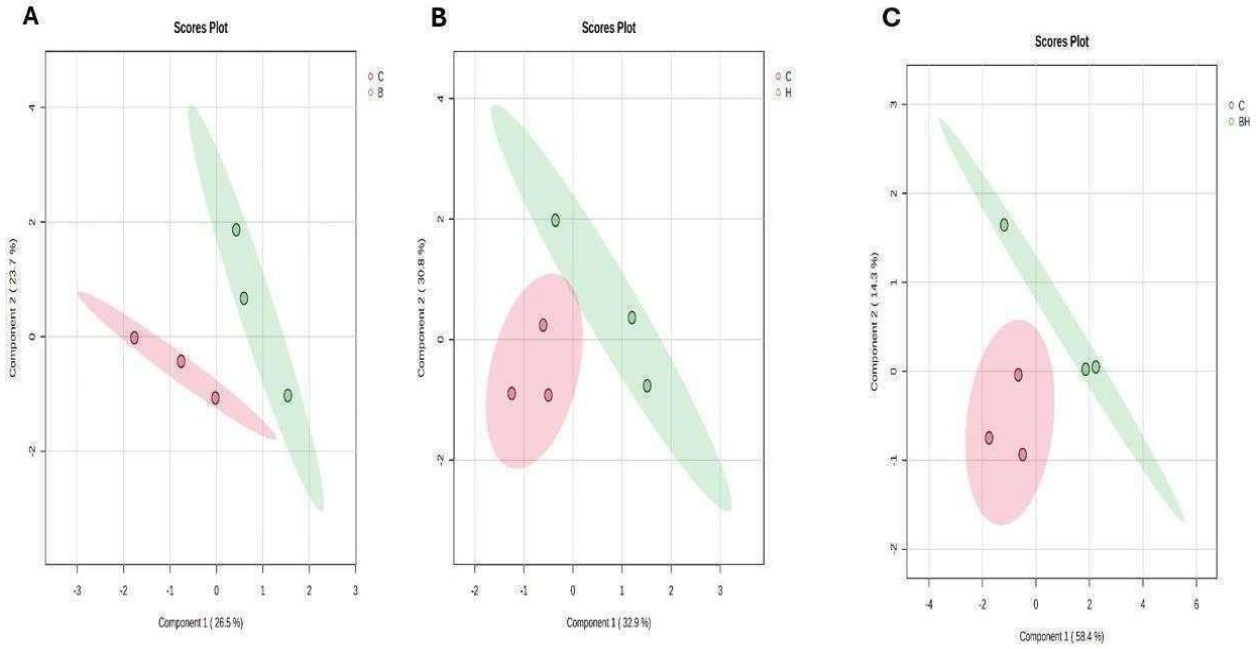

Figure 4.1: Score plots of the different treatment groups in tomatoes based on partial least square analysis (PLS-DA). (A) shows biostimulant control versus control, (B) shows heat shock versus control, and (C) shows biostimulant heat shock versus control. Samples were classified based on biostimulant and heat shock treatments, which are depicted in various colours.

4.3.2 Metabolic changes in amino acids, sugars, sugar alcohols, organic acids abundance during biostimulant and heat shock treatments

Heatmap hierarchical clusters were used to illustrate the abundance of the different metabolites across the four groups after the heat shock treatments in tomato leaf tissue. Although absolute values of metabolites were not determined, this would refer to changes in trends in abundance during the HS treatments in this study.

4.3.2.1 Amino acid metabolism during heat shock treatments

Amino acids are primarily involved in the building of proteins, growth regulation, stress response, signaling, and acting as precursors for various metabolites, essentially contributing to overall plant development and adaptation to environmental conditions (Trovato et al., 2021). An accumulation of amino acids in plants plays a crucial role in mitigating these effects by functioning as osmoprotectants, antioxidants, signaling molecules, and energy sources (Hammad & Ali, 2014; Naz et al., 2023).

The relative abundances of six amino acids across the four treatment groups (B, BH, C, H) were analyzed (Figure 4.2). The heatmap profile pattern suggests that the B and BH treatments were metabolically more similar, while the C and H treatments clustered separately, highlighting that application of the *E. maxima*-derived biostimulant modulated the metabolic responses. Under H treatment, valine, threonine and proline were more abundant compared to the C treatment. Proline is an osmoprotectant and a marker of osmotic stress and was also observed to increase in levels in the physiological assays (Chapter 3, Figure 3.7 B). Valine is a branched amino acid together with leucine and isoleucine. Branched amino acids are crucial in growth, stress adaption, and metabolic regulation, especially under abiotic stress such as HS and assist plants to withstand extreme temperature fluctuations (Xing & Last, 2017).

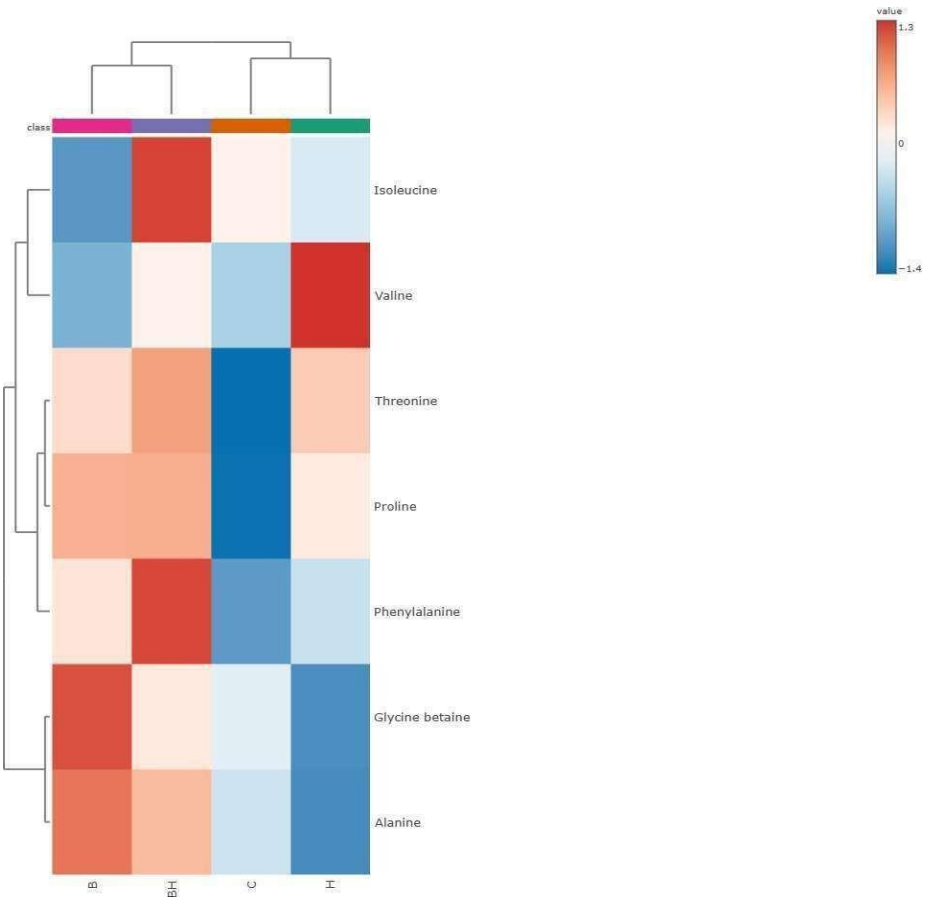

Figure 4.2: Heatmaps of differentially abundant amino acids in tomato leaf tissue across the four treatment groups, namely, C, control, B, biostimulant control, H, heat shock, and BH, biostimulant and heat shock. Distance measure (Euclidean), clustering method (ward), colour contrast (default). Significance was based on ANOVA Fisher's LSD with $P < 0.05$.

Under the B treatment, alanine, glycine betaine, phenylalanine, proline and threonine were more abundant compared to the C treatment (Figure 4.2). The BH treatment showed a similar clustering pattern to the B treatment with increased abundances for alanine, glycine betaine, phenylalanine, proline and threonine compared to the C treatment. This indicates that the prior application of the *E. maxima*-derived biostimulant induced a metabolic shift and supports priming of the metabolic response. Alanine, glycine betaine, phenylalanine, and proline play a crucial role in plant protection by maintaining osmotic balance, stabilizing photosynthesis, and enhancing antioxidant defence, ultimately improving plant growth and survival (Ayub, 2022; Agrawal, 2024; Hayat et al., 2012).

The SE *Kappaphycus alvarezii*-derived biostimulant has glycine betaine and cytokinin as key constituents. When applied to maize plants as a foliar spray under drought stress conditions it was found to enhance antioxidant activity and growth and yield of the maize plants (Trivedi, 2022). However, to date, there are no studies on whether *E. maxima* biostimulants have glycine betaine as a major constituent or whether the increase in abundance of glycine betaine was due to activation of associated metabolic pathways in tomato plants.

4.3.2.2 Sugar, and sugar alcohol metabolism during heat shock

Sugar and sugar alcohols function as energy sources to plants while also playing a crucial role in stress tolerance by acting as osmolytes, protecting cellular structures under adverse conditions such as HS (Bhattacharya & Kundu, 2020).

The relative abundances of eight sugars and sugar alcohols across the four treatment groups (B, BH, C, H) were analyzed (Figure 4.3). In response to HS stress, the H treatment had increases in the relative abundance of mannitol, glycerol, fructose, pinitol and glucose compared to the C treatment. Under abiotic stress such as HS, plants accumulate sugar alcohols such as mannitol, glycerol, and pinitol. These polyols act as osmoprotectants in mitigating oxidative stress by scavenging ROS and functioning as compatible solutes to maintain cell turgor (Meena et al., 2015). The increase in the abundance of the soluble sugars such as glucose and fructose could indicate the plants' tolerance to the HS treatments (Sami et al., 2016). It was observed that soluble sugars can be involved in ROS-producing metabolic pathways under stressful conditions such as HS (Couée, et al 2006). Sugars can feed into the oxidative pentose phosphate (Allan, et al 2023) pathway which results in NADPH required for regenerating antioxidants for ROS scavenging (Allan, et al 2023).

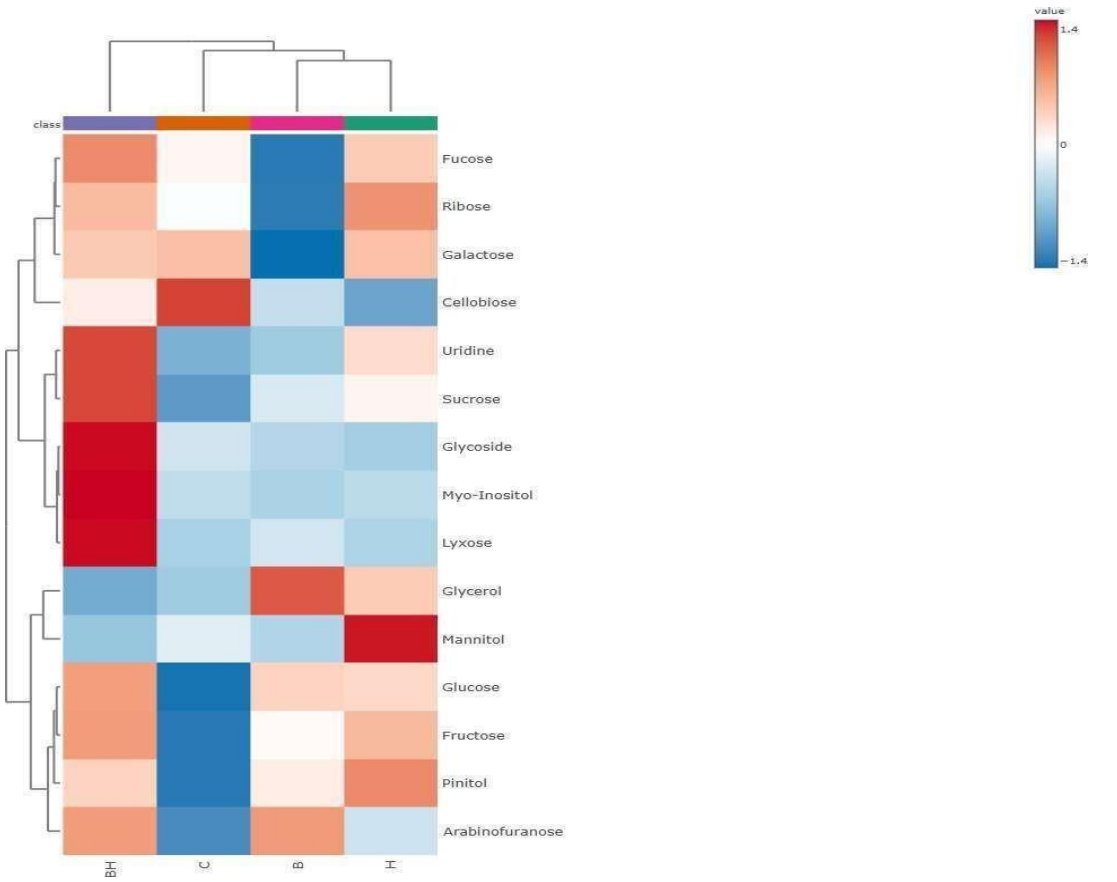

Figure 4.3: Heatmaps of differentially abundant sugars and sugar alcohols in tomato leaf tissue across the four treatment groups, where is C control, B biostimulant control, H heat shock, and BH biostimulant heat shock. Distance measure (Euclidean), clustering method (ward), colour contrast (default). Significance was based on ANOVA Fisher's LSD with $P < 0.05$.

Under the B and BH treatments, glucose, fructose, sucrose, myo-inositol, pinitol, and arabinofuranose had relative higher abundances compared to the C treatments (Figure 4.3). Again, this indicates that the application of the *E. maxima*-derived biostimulants resulted in a metabolic shift in the tomato plants. The heatmap clustering pattern also suggests that the B and BH treatments are metabolically more similar, while the C and H treatments are clustered separately, highlighting the biostimulant's potential modulatory effect on the HS response. The similar clustering pattern observed for B and BH may also indicate the same biochemical pathways being regulated by the biostimulant application.

The application of SEs has been linked to an increased accumulation of soluble sugars including glucose, fructose, and sucrose in plants under stress conditions (Kapur et al., 2018). This is due to SEs containing bioactive compounds that can enhance chlorophyll production

and photosynthetic efficiency, leading to an increased synthesis of carbohydrates, including sugars. The application of an *A. nodosum*-derived SE on sugarcane subjected to drought stress saw an increase in the accumulation of sucrose, resulting in the mitigation of drought stress and an increase in stalk and sugar yield. Leaf analysis confirmed that the SE application improved the metabolic activity, which led to an increase in sugars. In addition, the SE treated plants had increased antioxidant enzyme activity while MDA levels decreased (Jacomassi et al., 2022). As discussed above, sugars also help in maintaining cell turgor by balancing osmotic pressure, which is vital during heat stress and contribute to the OPP pathway for regeneration of antioxidants that neutralize ROS (Couée et al., 2006).

The application of the *E. maxima*-derived biostimulant results in the accumulation of soluble sugars under HS which may contribute to thermotolerance by acting as osmo-protectants, signaling molecules, carbon reserves and regulating various physiological processes by supporting antioxidant systems that neutralize ROS.

4.3.2.3 Organic acid metabolism during heat shock

Organic acids play a crucial role in plant physiology, helping in multiple ways such as growth promotion, nutrient uptake, stress tolerance, and defence mechanisms (Hathurusinghe, Azizoglu & Shin, 2024). They are produced as metabolic intermediates, which contribute to the regulation of cellular processes, energy production, and adaptive responses to environmental stressors (Igamberdiev & Bykova, 2018).

The relative abundances of seven organic acids across the four treatment groups (B, BH, C, H) were analyzed (Figure 4.4). It was noted that salicylic acid (SA), and alpha-ketoglutaric acid had higher relative abundance in the H treatment when compared to the C treatment. Alpha ketoglutaric acid enhances plant stress resistance by reducing oxidative stress markers and boosting antioxidant defences (Liu et al., 2024). However, under stressful conditions SA binds to the alpha ketoglutarate dehydrogenase enzyme to further reduce its activity, potentially affecting mitochondrial oxidative phosphorylation and electron transport chain components, this causes an excess in ROS accumulation and oxidative stress ultimately leading to cell death (Liao et al., 2015). It has been reported that higher concentrations of SA can inhibit shoot growth by affecting gene expression related to cell division, cell elongation, and hormone pathways (Meguro & Sato, 2014),. This inhibition may aid stress tolerance by activating antioxidants, secondary metabolites, and defence signaling pathways for protecting cellular integrity, ensuring metabolic stability, and improving plant thermotolerance (Choudhary et al., 2024).

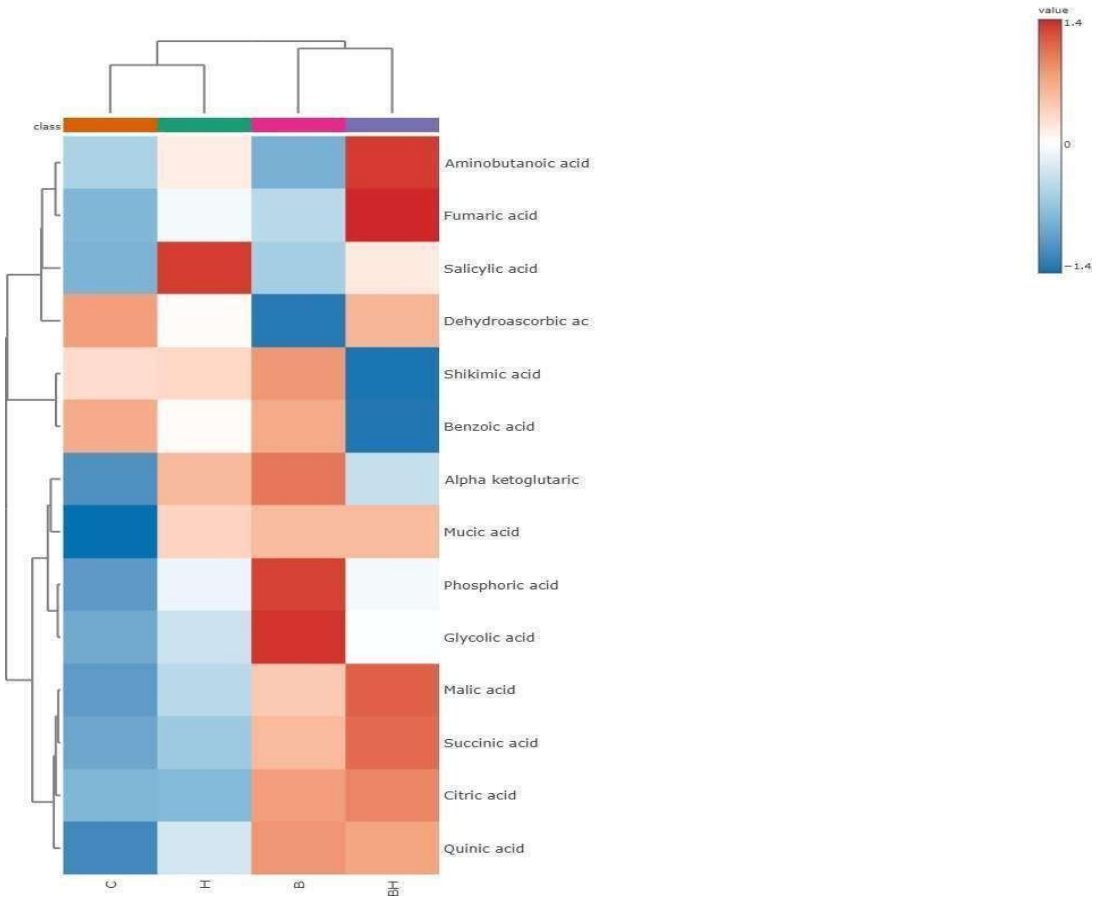

Figure 4.4: Heatmaps of differentially abundant organic acids in tomato leaf tissue across the four treatment groups, where C is control, B is biostimulant control, H is heat shock, and BH is biostimulant heat shock. Distance measure (Euclidean), clustering method (ward), colour contrast (default). Significance was based on ANOVA Fisher's LSD with $P < 0.05$.

In the B treatment, the organic acids, quinic acid, citric acid, succinic acid, malic acid, and phosphoric acid had higher relative abundances compared to the C treatment (Figure 4.4). The BH treatment showed a similar clustering pattern, with quinic acid, citric acid, succinic acid, malic acid, fumaric acid, amino butanoic acid (GABA) relative abundances higher compared to the C treatment (Figure 4.4). The clustering pattern also suggests that the B and BH treatments have similar metabolic responses, while the C and H groups are clustered separately, highlighting the biostimulant's potential to modulate the metabolic response to HS stress.

The listed organic acids play a crucial role in the TCA cycle, as they are central to energy-

generating pathways in the mitochondria, helping to produce ATP, NADH and $FADH_2$ to fuel various cellular processes (Chia et al., 2000). They promote plant growth by increasing chlorophyll content and mitigate stress (Chen et al., 2020).

They also assist in reducing the levels of ROS in plants by improving the antioxidant defence system (Kleiber et al., 2024), thereby increasing plant biomass (Chen et al., 2020).

Under stressful conditions, plants may experience TCA cycle inhibition, leading to energy deficiency and metabolic imbalances, and SE biostimulants can help in maintaining the TCA cycle to minimize these negative effects (Martínez-Reyes & Chandel, 2020). SEs provide bioactive compounds that enhance mitochondrial function and antioxidant enzyme activity to reduce ROS accumulation and facilitate carbon reallocation to stress tolerance pathways thereby improving plants resilience (Kumar et al., 2024). In a study investigated the metabolome of *A. thaliana* across different timepoints in response to SEs derived from *Durvillaea potatorum* and *Ascophyllum nodosum* and applied to roots (Tran et al., 2023) was observed that there is a strong accumulation of the TCA cycle metabolites and N-containing defensive metabolites such as glucosinolates indicating the enhancement of carbon and nitrogen metabolism including plant defence systems. The increased levels of organic acids not only support metabolic function but also aid in the stabilization of cellular structures and scavenging of ROS thereby reducing oxidative damage.

4.3.3 Metabolic pathway analysis for differentially abundant metabolites

Fold change analysis was done of the H, B and BH treatments relative to the C treatment. This was used to identify metabolites that were accumulating or decreasing in response to the specific treatments. Pathway analysis was used on all metabolites from fold change analysis using *A. thaliana* KEGG chassis as the pathway library related to the biological functions of identified metabolites. This revealed variations in metabolic pathways, suggesting different processes taking place during the HS treatments.

4.3.3.1 Metabolic pathways of amino acid analysis

4.3.3.1.1 H treatment versus C treatment pathway analysis

The metabolic pathways analysis of H treatment versus C treatment (Figure 4.5), shows three upregulated pathways. The valine, leucine and isoleucine biosynthesis, and glucosinolate biosynthesis pathways were upregulated in the H treatment with the pantothenate and CoA biosynthesis pathways being significantly upregulated. The amino acids produced included threonine, isoleucine, valine and phenylalanine. The observation that pantothenate and CoA

biosynthesis pathways involved in valine production were more upregulated under HS stress, correlates with our previous observations (Figure 4.2). This supports valine as a branched amino acid in potentially aiding in heat tolerance. Previous studies have confirmed that the accumulation of CoA regulates the metabolic flux into valine biosynthesis as an adaptive response to abiotic stress (Wang et al., 2020).

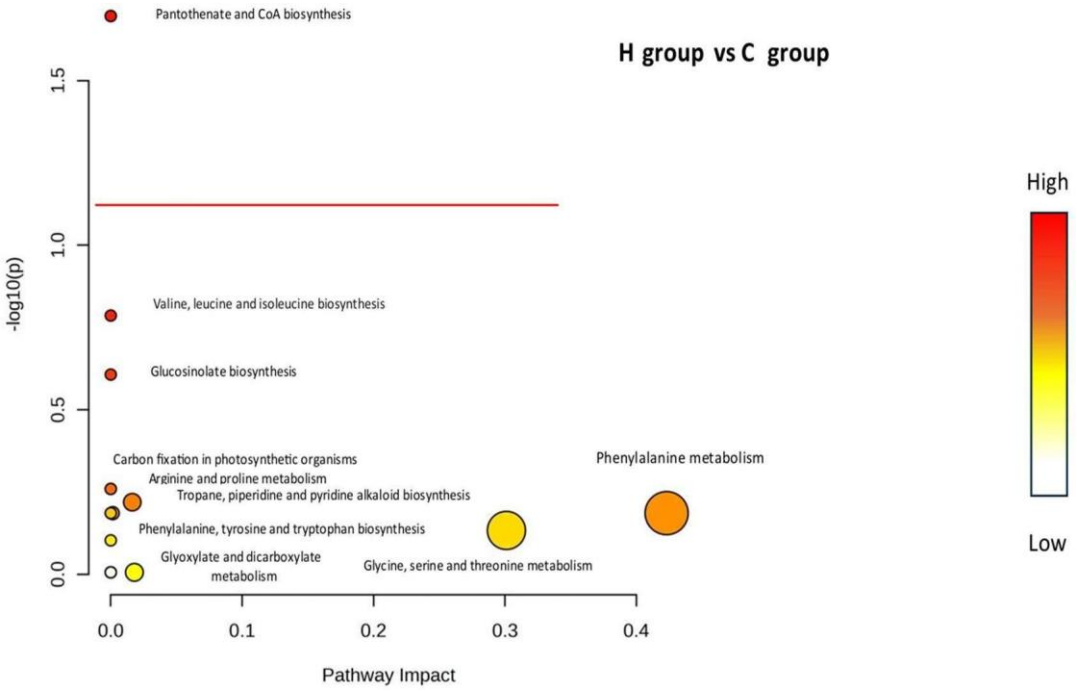

Figure 4.5: Metabolic pathway analysis graph on amino acid pathway impact of the biostimulant H treatment vs C treatment group samples identified in the different pathways. Significance was based on ANOVA Fisher's LSD with -log10(p) >1.3.

4.3.3.1.2 B and BH treatments vs C treatment pathway analysis

The metabolic pathways analysis of the B treatment versus C treatment (Figure 4.6), shows two upregulated pathways. Under this group, the pathways carbon fixation in photosynthetic organisms and glycine, serine and threonine metabolism pathway were found to be upregulated. The amino acids included glycine betaine and alanine.

The glycine, serine, and threonine metabolism pathway are crucial for glycine betaine biosynthesis. In the previous heatmap analysis of the B treatment (Figure 4.2), it was mentioned that glycine betaine plays a crucial role in plant protection by maintaining osmotic balance, stabilizing photosynthesis, and enhancing antioxidant capacity in plants. The use of *E. maxima*-derived SEs to prime tomato plants modulated glycine betaine biosynthesis for future stress responses.

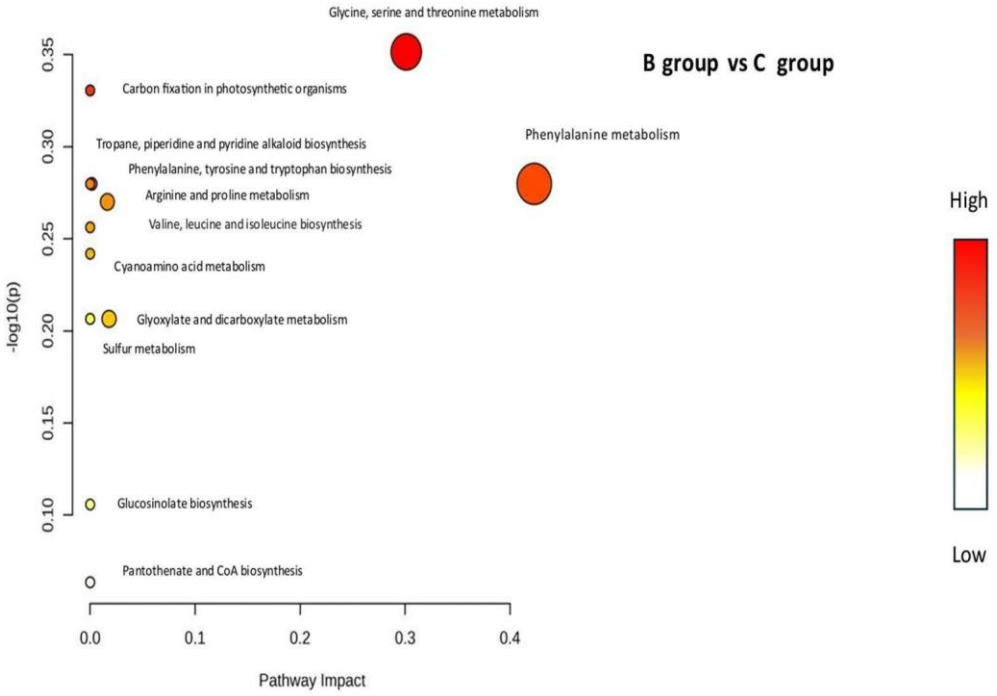

Figure 4.6: Metabolic pathway analysis graph on amino acid pathway impact of the biostimulant B treatment vs C treatment group samples identified in the different pathways. Significance was based on ANOVA Fisher's LSD with -log10(p) >1.3.

The metabolic pathways analysis of the BH treatment versus C treatment shows three upregulated pathways (Figure 4.7). These pathways included tropane, piperidine and pyridine alkaloid biosynthesis, phenylalanine metabolism, and phenylalanine, tyrosine and tryptophan biosynthesis. The upregulation of phenylalanine metabolism in the BH treatment correlated with the previous observations where there was an increase abundance of phenylalanine in the BH treatment (Figure 4.2). Phenylalanine serves as a precursor for wide range of secondary metabolites essentially opening metabolic pathways that lead to plant growth and defence against environmental stresses such as heat shock (Yamakawa & Hakata, 2010).

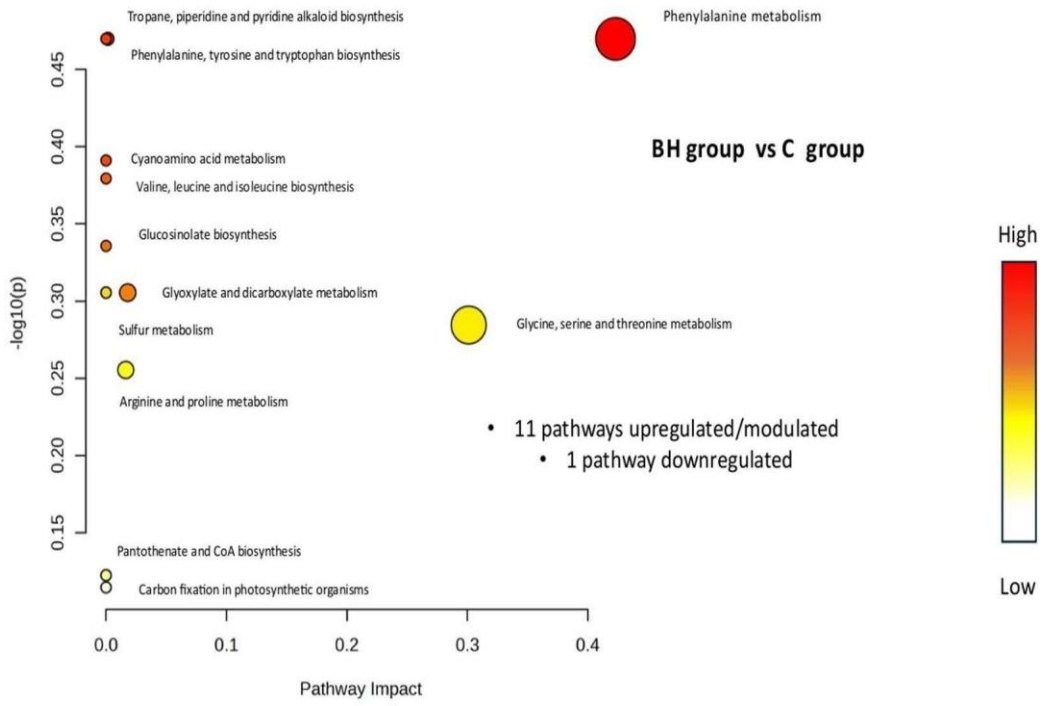

Figure 4.7: Metabolic pathway analysis graph on amino acid pathway impact of the biostimulant BH treatment vs C treatment group samples identified in the different pathways. Significance was based on ANOVA Fisher's LSD with -log10(p) >1.3.

4.3.3.2 Metabolic pathways of sugar and sugar alcohols analysis

4.3.3.2.1 H treatment versus C treatment pathway analysis

The metabolic pathways analysis of the H treatment versus C treatment included the upregulation of fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism, starch and sugar metabolism and galactose metabolism (Figure 4.8). The sugars produced from these pathways included glucose and fructose. The biosynthesis of soluble sugars correlated with our previous observations for increased abundance of sugars in the H treatment (Figure 4.3). We previously mentioned the role of soluble sugars as osmoprotectants, signaling molecules, carbon reserves and enabling antioxidant systems to neutralize ROS.

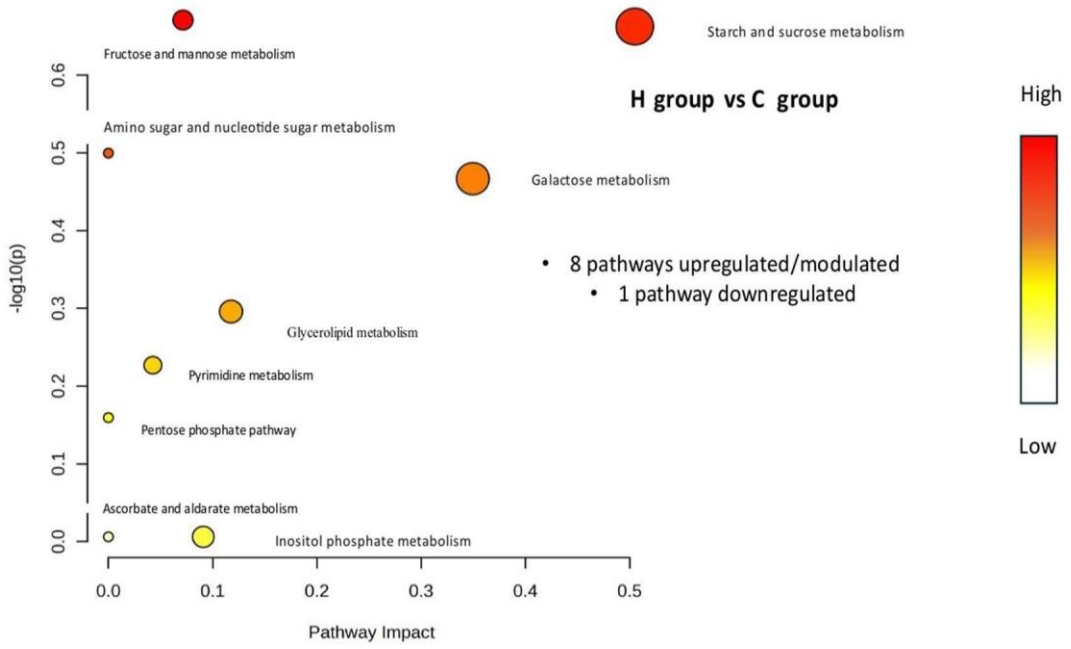

Figure 4.8: Metabolic pathway analysis graph on sugar and sugar alcohols pathway impact of the biostimulant H treatment vs C treatment group samples identified in the different pathways. Significance was based on ANOVA Fisher's LSD with -log10(p) >1.3.

4.3.3.2.1 B and BH treatments vs C treatment pathway analysis

The metabolic pathways analysis of the B treatment versus C treatment, included slight upregulation of starch and sucrose metabolism (Figure 4.9) while this pathway was more highly regulated in the BH treatment (Figure 4.10). This indicates a shift in the metabolic response when the *E. maxima*-derived biostimulant was applied without HS stress and a modulation of the same pathway with HS stress treatment. The sugars produced were fructose, and sucrose which were also observed previously (Figure 4.3). This also confirms that the application of SE resulted in increased accumulation of soluble sugars in tomato plants under HS stress conditions. This increased sugar accumulation could promote osmoprotection, energy supply, ROS scavenging, and stress signaling in the HS treatments.

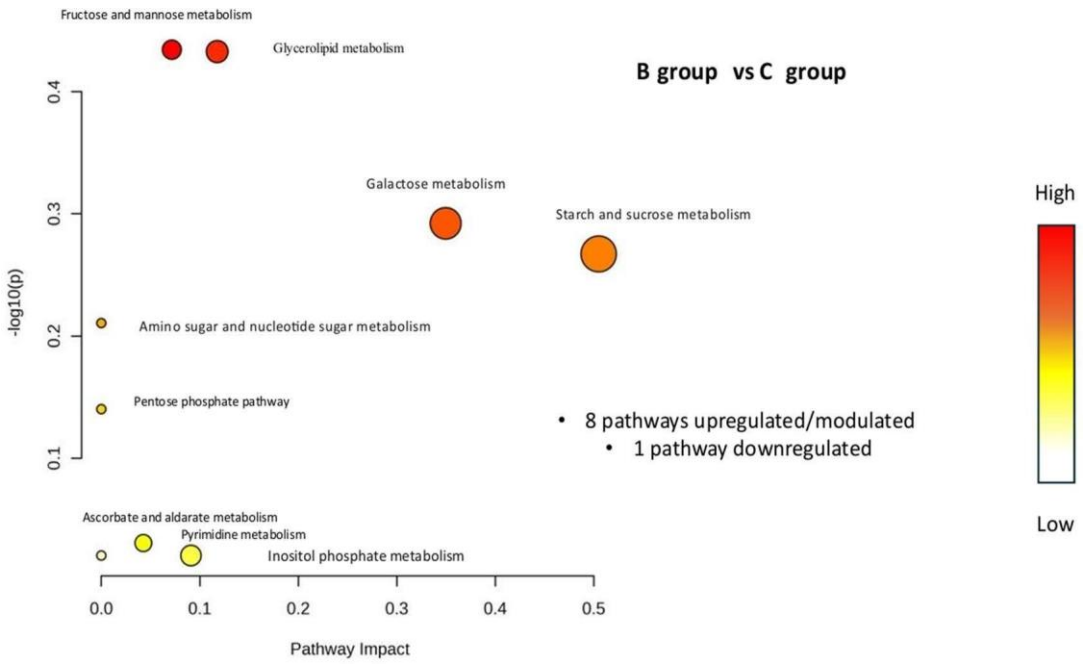

Figure 4.9: Metabolic pathway analysis graph on sugar and sugar alcohols pathway impact of the biostimulant B treatment vs C treatment group samples identified in the different pathways. Significance was based on ANOVA Fisher's LSD with -log10(p) >1.3.

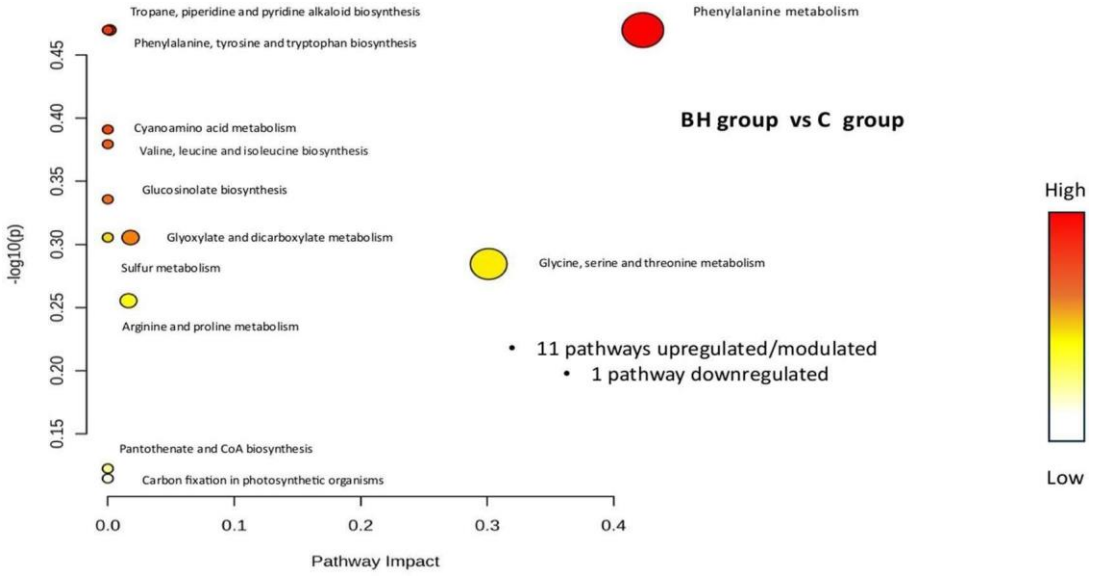

Figure 4.10: Metabolic pathway analysis graph on sugar and sugar alcohols pathway impact of the biostimulant BH treatment vs C treatment group samples identified in the different pathways. Significance was based on ANOVA Fisher's LSD with -log10(p) >1.3.

4.3.3.3 Metabolic pathways of organic acids analysis

4.3.3.3.1 H treatment versus C treatment pathway analysis

The metabolic pathways analysis of the H treatment versus C treatment (Figure 11) resulted in the upregulation of two pathways, the lipoic acid metabolism and butanoate metabolism. The organic acids produced under this treatment were Succinic acid and alpha ketoglutaric acid.

The observations of lipoic acid metabolism and butanoate metabolism which are involved in the biosynthesis of Succinic acid and alpha ketoglutaric acid correlates with our previous observation (Figure 4.4). this supports that both these organic acids are key intermediates to the TCA cycle, they play crucial roles in stress tolerance such as HS, regulating ATP production, ROS scavenging, nitrogen assimilation, and signaling pathways (Martínez-Reyes & Chandel, 2020).

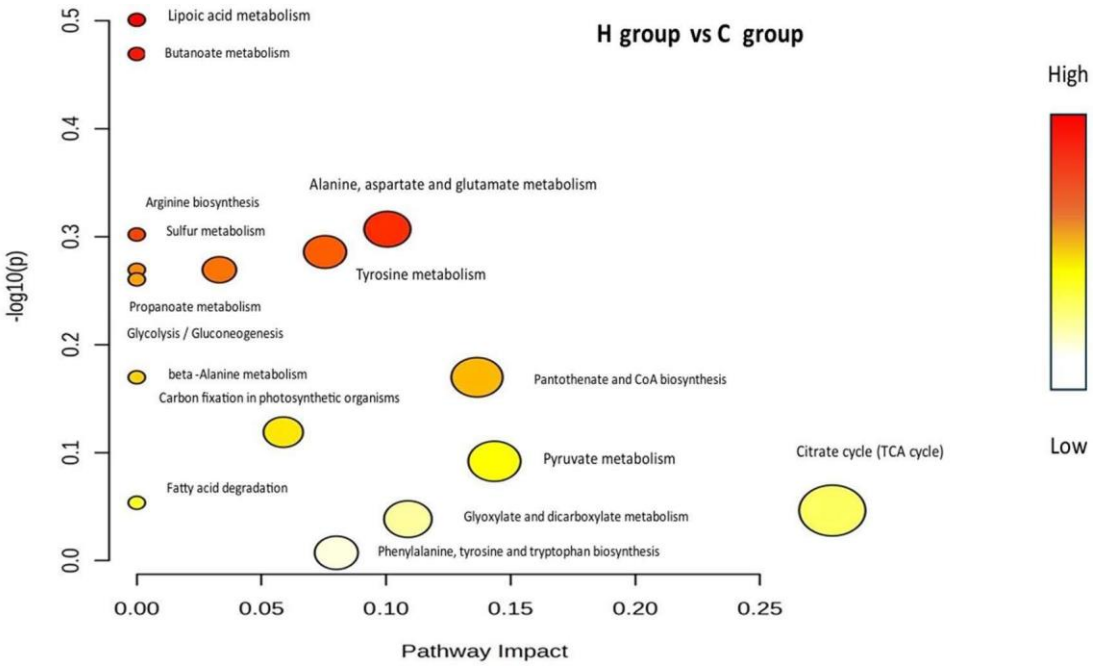

Figure 4.11: Metabolic pathway analysis graph on organic acids pathway impact of the biostimulant H treatment vs C treatment group samples identified in the different pathways. Significance was based on ANOVA Fisher's LSD with -log10(p) >1.3.

4.3.3.3.2 B and BH treatment vs C treatment pathway analysis

The metabolic pathway analysis of the B treatment versus C treatment pathways were found to be

slightly upregulated these included propanoate metabolism and sulfur metabolisms (Figure 4.12). The amino acid found in these 2 pathways was succinic acid, while these two pathways were significantly upregulated in BH treatment versus C treatment (Figure 4.13). This indicates a shift in metabolic response when the *E. maxima*-derived biostimulant was applied without HS stress and a modulation of the same pathway with HS stress treatment. This also confirms that SEs resulted in the accumulation of the TCA cycle intermediates, improving plant resilience under HS treatments.

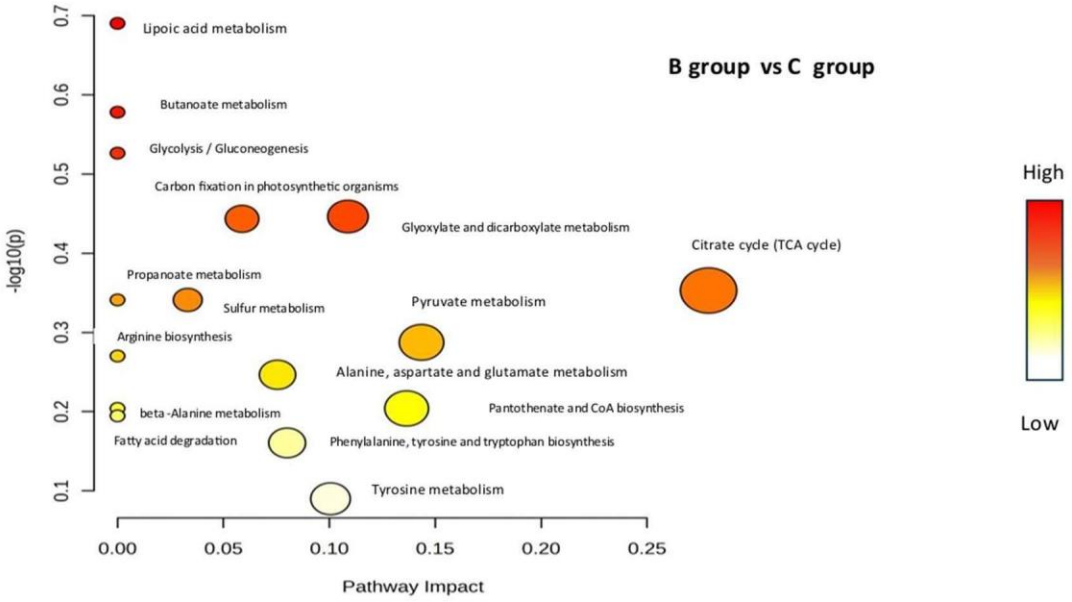

Figure 4.12: Metabolic pathway analysis graph on organic acids pathway impact of the biostimulant B treatment vs C treatment group samples identified in the different pathways. Significance was based on ANOVA Fisher's LSD with -log10(p) >1.3.

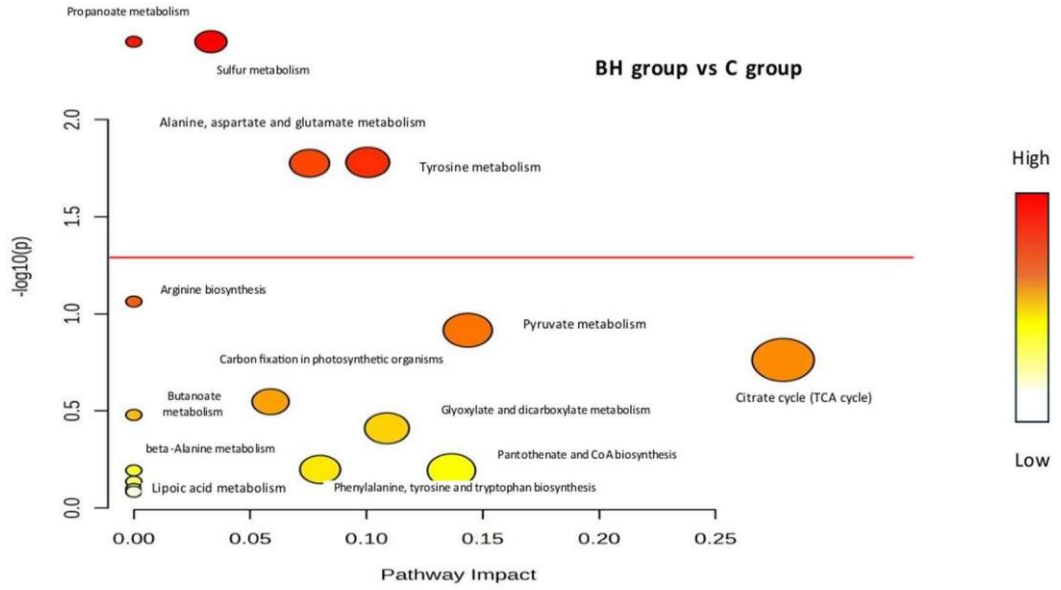

Figure 4.13: Metabolic pathway analysis graph on organic acids pathway impact of the biostimulant BH treatment vs C treatment group samples identified in the different pathways. Significance was based on ANOVA Fisher's LSD with -log10(p) >1.3.

4.4 Conclusions

This chapter aimed to investigate the effects of *E. maxima*-derived biostimulant on tomato plants subjected to HS treatments, with a focus to use GC-MS metabolomics approach to investigate the specific amino acids, sugars, sugars alcohols, and organic acids that were abundant or present when the tomato plants were primed prior with the SE based biostimulant before HS treatment. The PLS-DA treatment groupings across the heat treatments suggest that the metabolites in tomatoes leaf material varied significantly between the four treatment group, particularly the BH treatment versus C treatment, showing a prominent separation along the first component.

The heatmap hierarchical clusters for the B and BH treatment showed that alanine, glycine betaine, phenylalanine, proline and threonine were more abundant compared to the C treatment indicating that prior application of the *E. maxima*-derived biostimulant induced a metabolic shift and supports priming of the metabolic response. Additionally, the sugar and sugar alcohols for the B and BH treatments, glucose, fructose, sucrose, myo-inositol, pinitol, and arabinofuranose had relatively higher abundances compared to the C treatments. Similarly, this indicated that the application of the *E. maxima*-derived biostimulants resulted in a metabolic shift in the tomato plants. The application of SEs has been linked to an

increased accumulation of soluble sugars including glucose, fructose, and sucrose in plants under stress conditions (Kapur et al., 2018).

In organic acids, the B treatment had higher relative abundances compared to the C treatment or to what quinic acid, citric acid, succinic acid, malic acid, and phosphoric acid had. The BH treatment showed a similar clustering pattern, with quinic acid, citric acid, succinic acid, malic acid, fumaric acid, amino butanoic acid (GABA) relative abundances being higher compared to the C treatment. The listed organic acids play a crucial role in the TCA cycle, as they are central to energy-generating pathways in the mitochondria, helping to produce ATP, NADH and FADH2 to fuel various cellular processes.

Pathway analysis results validated the observations of the heatmap results therefore suggesting that SEs potentially increase the accumulation of metabolites in tomato plants under HS stress conditions. In conclusion, we showed that the *E. maxima*-derived biostimulant acts as a priming agent that can influence various metabolic pathways, leading to the accumulation of metabolites that are crucial for stress tolerance. This metabolic reprogramming aids in better adaptation to HS conditions.

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Chapter 5

Conclusions and future work

5.1 Conclusions

The brown algae, *E. maxima* found mainly along the southern Atlantic coast of Africa (Anderson et al., 2003) is an important commercial source for the production of alginate, animal feed, and plant biostimulants. The growth promoting effects of *E. maxima* SE on different crops has been attributed to different growth regulators including auxins, cytokinins, polyamines, gibberellins, abscisic acid, and brassinosteroids, found in the extract (Papenfus et al., 2013).

While certain seaweed species have been shown to reduce heat stress when used as biostimulants (Ertani et al., 2018; Goñi et al., 2018; MacKinnon et al., 2010; Omidbakhshfard et al., 2020; Rayirath et al., 2009; Shukla et al., 2019), no study has yet evaluated the effects of a commercial *E. maxima*-derived biostimulant in alleviating heat shock stress in tomatoes or explored its underlying molecular mechanisms.

This aim of this study was to determine whether prior foliar application of *E. maxima* biostimulant on tomato plants leaves assisted in enhancing the thermotolerance of tomatoes to heat shock stress and to characterise the physiological and metabolomic response.

The first objective of this study was to examine physiological, morphological, and biochemical changes in tomato plants pre-treated with the *E. maxima* biostimulant and exposed to 4 hours of heat shock, followed by assessments such as electrolyte leakage, chlorophyll content, and antioxidant enzyme activity.

In the morphological experiments, shoot and root lengths were measured. No notable differences were observed in root morphology across treatment groups, including after heat shock (HS), likely because both water and *E. maxima*-based biostimulant were applied as foliar sprays rather than soil drenches. Additionally, the short, two-day treatment period may have been insufficient to affect root structure. Tomato shoot morphology was also evaluated. The heat-stressed (H) group showed visible symptoms such as leaf drooping, curling, and stem bending typical plant responses to high temperatures aimed at reducing moisture loss. In contrast, the biostimulant + heat (BH) group showed no such symptoms; leaves remained green, upright, and intact, indicating that pre-treatment with the *E. maxima* biostimulant enhanced shoot thermotolerance. These findings align with Zhang et al. (2023), who reported that foliar application of an *A. nodosum*-derived biostimulant improved turf quality, leaf color, and chlorophyll content in heat- and drought-stressed *Agrostis stolonifera*. Electrolyte leakage analysis showed that *E. maxima*-treated plants under heat stress (BH) experienced minimal

membrane damage, with significantly lower leakage compared to the C and B groups. This suggests the biostimulant primed the plants for improved heat tolerance.

Under light-adapted conditions (ΔF/Fm'), the BH group showed significantly higher photosynthetic efficiency than the H group, indicating that *E. maxima* priming helped preserve PSII function during heat stress. A similar pattern was observed under dark-adapted conditions (F_v/F_m), with BH values approaching those of the control group, further supporting the stated biostimulant's protective effect. Chlorophyll a and b levels increased in the B group compared to the C and H, groups with the BH group showing significantly higher levels than the H groups. This indicates that pre-treatment with the biostimulant mitigated chlorophyll loss due to heat stress. These results are supported by Makonya et al. (2025), who found that an *A. nodosum* extract enhanced thermotolerance in raspberries by maintaining chlorophyll fluorescence (Fv/Fm) and photosynthesis under heat stress. Similarly, the BH group in this study maintained photosynthetic efficiency close to control levels, confirming the biostimulant's protective role.

Biochemical experiments measured lipid peroxidation (MDA), proline, DPPH, and FRAP in tomato leaves after heat shock (HS) treatments. As expected, MDA levels were highest in the H treatment and interestingly in the B treatment when compared to the control. The observed MDA increase in the B treatment indicates increased ROS and oxidative stress and may suggest a stress priming effect. This is supported by the BH treatment which had significantly reduced MDA levels compared to the H and B treatments, suggesting that prior application of *E. maxima*- derived biostimulant enhances thermotolerance and prepares plants for oxidative damage induced by heat stress.

The H treatment had significantly higher proline levels, indicating oxidative stress. The BH treatment had proline levels similar to the C treatment, suggesting that *E. maxima*-priming reduced the stress response to heat shock. Previous studies suggest that SE biostimulants reduce proline accumulation by either mimicking its protective role or enhancing other stress tolerance mechanisms (Lefi et al., 2023). The reduction in the accumulation of proline in plants is not a guaranteed response. In other studies, biostimulants promote proline accumulation, particularly when plants are subjected to abiotic and biotic stresses (Goñi, Quille & O'Connell, 2018). It was hypothesized that the difference in proline accumulation kinetics in response to seaweed application might be linked to the type of biostimulant used, crop class, or application type (Goñi, Quille & O'Connell, 2018).

The H treatment showed the lowest DPPH levels, indicating oxidative stress and ROS

accumulation. The B treatment had higher DPPH levels than the C treatment which indicate that antioxidant systems modulate ROS homeostasis for normal plant growth and development and subsequent stress. The BH treatment had the highest DPPH, suggesting that the *E. maxima* biostimulant enhanced antioxidant capacity to mitigate HS effects. In the FRAP assay both the B and BH treatments had similar FRAP levels, lower than H but not significantly. The BH treatment had increased FRAP compared to the C treatment, indicating that prior *E. maxima* application primed antioxidant systems for better heat shock tolerance. Although FRAP and DPPH assays assess antioxidant activity, the higher FRAP in H treatment suggests an increase in certain antioxidants, while the lower DPPH indicates that they may not effectively neutralize free radicals.

The second objective of the study was to identify specific metabolites (amino acids, sugars, sugar alcohols, organic acids) that change in abundance in tomato plants treated with the *E. maxima* biostimulant under HS conditions, providing insights into the priming mechanism for heat shock stress tolerance. Partial least squares discriminant analysis (PLS-DA) identified key features differentiating the treatment groups in metabolomics data. The B vs. C, H vs. C, and BH vs. C treatments showed distinct separation. For B vs. C, components 1 and 2 explained 26.5% and 23.7% of the variation, indicating moderate separation. H vs. C had stronger separation, with components 1 and 2 explaining 32.9% and 30.8%, respectively. BH vs. C showed prominent separation, with component 1 explaining 58.4% of the variation. These results highlight significant metabolic differences across the treatment groups.

Heatmap clustering revealed the abundance of metabolites in tomato leaves. For amino acids, B treatment showed higher levels of alanine, glycine betaine, phenylalanine, proline, and threonine compared to C treatment. BH treatment displayed similar clustering, with increased levels of these amino acids. With respect to sugars and sugar alcohols, the B and BH treatments had higher levels of glucose, fructose, sucrose, myo-inositol, pinitol, and arabinofuranose compared to the C treatment, with B and BH treatments showing similar metabolic patterns. Organic acids like quinic acid, citric acid, succinic acid, and malic acid were more abundant in the B and BH treatments and play a crucial role in the TCA cycle, as they are central to energy-generating pathways in the mitochondria, helping to produce ATP, NADH and FADH2 to fuel various cellular processes. They promote plant growth by increasing chlorophyll content and mitigating stress.

They also assist in reducing the levels of ROS in plants by improving the antioxidant defence system. Metabolic pathway analysis confirmed the heatmap findings, showing increased

metabolite accumulation in tomato plants under HS stress with *E. maxima* SE treatment.

In summary our results provide evidence that the E. *maxima* derived biostimulant when applied as a foliar spray to tomato plants acts as a priming agent to improve thermotolerance to subsequent heat shock stress as observed by changes in morphological, physiological, biochemical and metabolomic responses.

5.2 Future work

The context of this study was to investigate heat shock stress as climate change is expected to increase the frequency and duration of heatwaves, putting crops at greater risk of sudden temperature spikes. Future experiments should focus on heat stress rather than heat shock, as this would provide more ecologically relevant insights into plant responses. Heat shock causes immediate damage, which does not reflect how plants recover over time. Additionally, the full potential of biostimulants might not be realized under heat shock conditions. Biostimulant treatments should be extended beyond a week, as some biostimulants work by gradually enhancing stress tolerance. Prolonged treatment may also support root development and improve nutrient acquisition over time.

In addition to metabolomics, future studies should incorporate multi-omics approaches, including transcriptomics, proteomics, and phenolic profiling, to gain a comprehensive understanding of the molecular mechanisms behind biostimulant effects. This would help identify key genes, pathways, and protein-level changes that influence stress signaling and plant growth.

5.3 References

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